

Investigating the altered human metabolome induced by a marathon and the recovery thereof

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“All of science is nothing more than the refinement of
everyday thinking.”

~Albert Einstein

TABLE OF CONTENTS

ACKNOWLEDGEMENTS	IV
SUMMARY	V
LIST OF TABLES.....	VII
LIST OF FIGURES.....	IX
LIST OF ABBREVIATIONS	XI
CHAPTER 1: PREFACE	1
1.1. BACKGROUND AND MOTIVATION	1
1.2. AIMS AND OBJECTIVES	2
1.2.1. Aims	2
1.2.2. Objectives.....	2
1.2.3. Supporting objectives	3
1.3. STRUCTURE OF THESIS AND RESEARCH OUTPUTS.....	3
1.4. AUTHOR CONTRIBUTIONS	4
1.5. REFERENCES	6
CHAPTER 2: LITERATURE REVIEW	8
2.1. INTRODUCTION	8
2.2. PHYSIOLOGICAL (STRUCTURAL) AND IMMUNOLOGICAL BIOMARKER ADAPTATIONS	9
2.2.1. Blood biochemistry	9
2.2.2. Cardiovascular markers	21
2.2.3. Muscle, renal and hepatic damage markers.....	23
2.2.4. Immune response and inflammation	25
2.2.5. Structural damage and associated hormones	27
2.3. METABOLOMICS AND METABOLOMIC BIOMARKERS.....	29
2.3.1. Carbohydrate metabolism and the tricarboxylic acid (TCA) cycle	40
2.3.2. Lipid metabolism.....	41
2.3.3. AA metabolism.....	43
2.3.4. Purine metabolism, ROS and vitamins.....	45
2.4. IN A NUTSHELL, WHAT DOES THIS MEAN?.....	46
2.4.1. The problem and its roots	46
2.4.2. A possible proficiency classification system.....	46

2.5. RECOVERY.....	48
2.5.1. The basics of unaided metabolic recovery	48
2.5.2. Recovery modality aided post-exercise recovery	49
2.6. REFERENCES	51
CHAPTER 3: METHODOLOGY AND MATERIALS	65
3.1. INTRODUCTION	65
3.2. EXPERIMENTAL DESIGN	65
3.2.1. Ethical approval.....	66
3.2.2. Participants	67
3.2.3. The Druridge Bay Marathon and supplements.....	67
3.2.4. Clinical sample collection and storage	68
3.2.5. Quality control samples	68
3.2.6. Total metabolome extraction and derivatisation	69
3.2.7. GCxGC-TOFMS analysis and data processing	69
3.2.8. Data clean-up, quality assurance, and statistical analyses.....	70
3.3. REFERENCES	71
CHAPTER 4: MARATHON-INDUCED METABOLIC ADAPTATIONS	73
4.1. ABSTRACT	73
4.2. INTRODUCTION	74
4.3. MATERIALS AND METHODS	75
4.3.1. Participants and clinical samples.....	75
4.3.2. Sample analysis	75
4.3.3. Data clean-up and statistical analyses	76
4.4. RESULTS.....	76
4.5. DISCUSSION.....	79
4.6. CONCLUSION	84
4.7. LIMITATIONS AND FUTURE PROSPECTS	84
4.8. REFERENCES	84
CHAPTER 5: UNAIDED METABOLIC RECOVERY OF ATHLETES	89
5.1. ABSTRACT	89
5.2. INTRODUCTION	90
5.3. METHODS AND MATERIALS	91
5.3.1. Participants and clinical samples.....	91
5.3.2. Sample analysis	91
5.3.3. Data clean-up and statistical analyses	91
5.4. RESULTS.....	92

5.4.1. Multivariate statistical outputs	92
5.4.2. Univariate statistical outputs	94
5.5. DISCUSSION.....	96
5.5.1. Carbohydrate metabolism.....	96
5.5.2. Lipid metabolism.....	97
5.5.3. Ketone body production and the TCA cycle	98
5.5.4. AA metabolism.....	99
5.6. CONCLUSION	101
5.7. LIMITATIONS AND FUTURE PROSPECTS	101
5.8. REFERENCES	102
CHAPTER 6: BEETROOT JUICE AIDED METABOLIC RECOVERY OF ATHLETES	105
6.1. ABSTRACT	105
6.2. INTRODUCTION	106
6.3. METHODS AND MATERIALS	107
6.3.1. Participants and clinical samples.....	107
6.3.2. Sample analysis	108
6.3.3. Data processing and statistical analyses	108
6.4. RESULTS	109
6.5. DISCUSSION.....	112
6.6. CONCLUSION	114
6.7. LIMITATIONS AND FUTURE PROSPECTS	114
6.8. REFERENCES	115
CHAPTER 7: CONCLUSION AND FUTURE PROSPECTS.....	118
7.1. CONCLUDING REMARKS	118
7.2. FUTURE PROSPECTS.....	119
ANNEXURE A.....	121
ANNEXURE B.....	128
ANNEXURE C.....	130

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“Great things are not done by one person. They are done by a team of people.”

~ Steve Jobs

SUMMARY

Although moderate physical activity is substantially beneficial to human health, continuous participation in endurance running (≥ 5 km), has been associated with various adverse physiological and immunological effects. Despite these effects, endurance running is still considered a popular global pass time that has rapidly evolved into a highly competitive sport, classified as either a half-marathon (21 km), marathon (42 km) or ultra-marathon (≥ 42 km). Considering that recovery is a key component in athletic performance, previous research approaches have been directed towards identifying more cost-effective, readily available recovery approaches, such as functional foods (beetroot, cherry, pomegranate, bananas, etc.). These foods, especially beetroot, are rich in antioxidants and possess potent radical scavenging properties, that may aid in the physiological and immunological post-training recovery of athletes. Despite the well-characterised nature of the aforementioned effects and the recovery thereof, very limited literature exists pertaining to the holistic metabolic adaptations that may arise due to participation in endurance races and how these may recover with and without the intervention of recovery aids. Since metabolomics not only elucidates the effects of an intervention at metabolic level, but also provides a representation or prediction of the organism's phenotypic state at a specific point in time, it is an excellent research approach to fill these voids. Metabolomics is defined as the comprehensive identification and quantification of the small metabolites (<1500 Da) involved in the metabolic reactions associated with cell growth and function in a biological sample, using high-throughput separation systems, in conjunction with various statistical approaches.

As such, the current investigation aimed to: (1) better characterise the metabolic adaptations induced by a marathon, (2) investigate the unaided or natural metabolic recovery trend thereof, and (3) determine whether beetroot juice ingestion may expedite this recovery process in athletes within 48 h post-marathon. For this double-blinded placebo-controlled study, serum samples from 31 athletes were obtained 24 h before (pre-marathon), immediately after, as well as 24 h and 48 h after completing the Druridge Bay Marathon. During the post-race recovery period (i.e. 24 h and 48 h post-marathon), these athletes were subdivided into beetroot ($n=15$) and placebo ($n=16$) ingesting cohorts, based on the supplement received ($\pm$ three times a day). All samples were extracted using a total metabolome extraction and analysed using an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry-based metabolomics approach.

Based on the comparison of the pre-marathon and immediately post-marathon serum metabolite profiles of athletes ($n=31$), it was clear that a marathon induces a metabolic shift between various fuel substrate pathways, as evident by the elevated concentrations of carbohydrates, fatty acids, tricarboxylic acid cycle intermediates and ketones, along with a reduction in amino acids. Additionally, elevated odd-chain fatty acids and α -hydroxy acids indicated the utilisation of α -oxidation and autophagy as alternative energy-producing mechanisms. Adaptations in various gut microbe-associated markers were also observed and correlated with the metabolic flexibility of the athlete.

Surprisingly, when investigating the unaided metabolic recovery trend of placebo-ingesting athletes (n=16; comparing immediately post-marathon, 24 h and 48 h post-marathon profiles), the metabolic data indicated recovery to pre-marathon-related state within 24 h post-marathon, with the exception of xylose which recovered within 48 h. This may be explained by a reduction in energy requirements during recovery and the subsequent downregulation of fuel substrate catabolism, resulting in the activation of glycogenesis, uridine-dependent nucleotide synthesis, protein synthesis, and the reduction of cellular autophagy.

Finally, when determining the effects of beetroot juice supplementation on the recovery of athletes (n=15; comparing immediately post-marathon, 24 h and 48 h post-marathon profiles), a similar recovery trend to that of the placebo cohort was observed. Although a few significant, albeit small, intervention and/or interaction associated metabolic fluctuations were identified between the cohorts, the majority of these presented a considerable amount of post-marathon inter-cohort variation. As such, only metabolites with a large practical significance within the recovery period was considered to be pertinent in determining the efficacy of beetroot juice supplementation. These metabolites (n=4) were predominantly associated with beetroot constituents and the microbial fermentation thereof, with little to no apparent value to the immediate metabolic recovery of the athletes. Considering this, and the global metabolic recovery trends of the two opposing cohorts, beetroot ingestion did not expedite the metabolic recovery process of these athletes within 48 h post-marathon.

Keywords: Marathon; Athletes; Untargeted metabolomics; Fuel substrates; Unaided recovery; Beetroot juice

LIST OF TABLES

Chapter 1:

<i>Table 1-1: Academic contributions of all parties involved in this thesis.</i>	<i>5</i>
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Chapter 2:

<i>Table 2-1: ER-induced physiological and immunological-associated biomarker adaptations reported in recent (2000-2020) literature (arranged according to publication year).</i>	<i>10</i>
--	-----------

<i>Table 2-2: ER-induced metabolic biomarker adaptations reported in recent (2000–2020) literature, arranged according to the publication year.....</i>	<i>31</i>
---	-----------

<i>Table 2-3: Proposed proficiency classification system for ER investigations.....</i>	<i>47</i>
---	-----------

Chapter 3:

<i>Table 3-1: A summary of the participant characteristics of the placebo and beetroot ingesting cohorts.</i>	<i>67</i>
--	-----------

<i>Table 3-2: Macro-nutrient composition of the beetroot juice and placebo supplements provided to athletes [adapted from Clifford et al. (2017)].</i>	<i>68</i>
---	-----------

Chapter 4:

<i>Table 4-1: Summary of the participant demographic information</i>	<i>75</i>
--	-----------

<i>Table 4-2: The significant serum metabolite markers best describing the variation between the pre- and post-marathon groups, listed alphabetically.</i>	<i>77</i>
---	-----------

Chapter 5:

<i>Table 5-1: Ratio of concentration fluctuations in the serum metabolite markers (n=61) best describing the detected variation and metabolic recovery of the athletes within 48 h post-marathon.....</i>	<i>94</i>
---	-----------

Chapter 6:

<i>Table 6-1: Metabolites varying significantly between the beetroot and placebo cohorts, according to RM ANOVA and day-specific pairwise comparisons.....</i>	<i>111</i>
--	------------

Annexure A:

<i>Table A-1: Participant characteristic and performance distribution in relation to the entire cohort</i>	<i>123</i>
--	------------

Table A-2: Serum metabolite markers (n=61) best describing the detected variation and metabolic recovery of the athletes within 48 h post-marathon. 124

Annexure B:

Table B-1: Average concentrations and RM ANOVA p-values the metabolites selected to be significant pertaining to the aim of this investigation..... 129

LIST OF FIGURES

Chapter 3:

- Figure 3-1: Schematic representation of the overall experimental design used to address the aims and objectives of this metabolomics investigation. 66
- Figure 3-2: Principal component analysis scores plot of the obtained serum samples (a) before and (b) after removal of the outlier quality control samples. 71

Chapter 4:

- Figure 4-1: Multilevel principal component analysis scores plot showing clear differentiation of the serum samples of marathon athletes before (denoted by circles) and after (denoted by squares) the completion of a marathon. The variance accounted for are indicated in parenthesis..... 76
- Figure 4-2: A schematic representation of the altered serum metabolome induced by a marathon. The altered metabolites are either denoted as increased (↑) or decreased (↓) relative to the pre-marathon group..... 83

Chapter 5:

- Figure 5-1: Schematic representation of the sample group comparisons performed during this investigation 92
- Figure 5-2: The ASCA plot indicating the natural, time-dependent differentiation of the comparative groups: Pre-marathon (denoted by blue/circle), post-marathon (denoted by pink/square), 24 h post-marathon (denoted by turquoise/right-tilted triangle) and 48 h post-marathon (denoted with black/left-tilted triangle). The variance accounted for by each latent variable (LV) is indicated in parenthesis on the respective axes. The ellipsoids represent 95% confidence intervals of each time points centroid. 93
- Figure 5-3: A metabolic chart summarising the major pathways affected during and after a marathon perturbation (adapted from Stander et al. (2018)). Microbial interactions are denoted with bold dashed arrows and the line-graphs schematically illustrate the time-dependent concentration shifts of the significantly altered metabolites..... 100

Chapter 6:

- Figure 6-1: Multi-level principal component analysis plot of the pre-marathon serum metabolite profiles of the beetroot group relative to the (a) 24h post-marathon and (b) 48h post-marathon metabolite profiles, profiles of the same group 109

Figure 6-2: Unfolded principal component analysis (a) with, and (b) without confidence intervals, depicting the global effect beetroot and placebo aided recovery over time 110

Annexure A:

Figure A-1: The larger scope of this investigation consists of multiple objectives: Objective 1 (Chapter 4): Effects of a marathon on the serum metabolome of athletes (n=31); Objective 2 (Chapter 5): Metabolic recovery without the intervention of recovery aids, by comparing pre-, post- as well as 24 h and 48 h post-marathon samples of the athletes that ingested placebo supplements (n=16); Objective 3 (Chapter 6): Effect of beetroot juice supplementation on metabolic recovery, by comparing pre-, post- as well as 24 h and 48 h post-marathon samples of the athletes that ingested beetroot juice supplements (n=15). 121

Figure A-2: Multilevel principal component analysis plots of the comparative groups: (a) Pre-marathon (denoted by blue/circle) vs post-marathon (denoted by pink/square); (b) post-marathon (denoted by pink/square) vs 24 h post-marathon (denoted by turquoise/triangle); (c) post-marathon (denoted by pink/square) vs 48 h post-marathon (denoted by black/triangle); (d) pre-marathon (denoted by blue/circle) vs 24 h post-marathon (denoted by turquoise/triangle); (e) pre-marathon (denoted by blue/circle) vs 48 h post-marathon (denoted by black/triangle); (f) 24 h post-marathon (denoted by turquoise/right-tilted triangle) vs 48 h post-marathon (denoted by black/left-tilted triangle) 122

Figure A-3: Dendrogram metabolome clustering of athletes at the various comparative timepoints: (a) Pre-marathon vs post-marathon, (b) post-marathon vs 24 h post-marathon, (c) post-marathon vs 48 h post-marathon, (d) 24 h vs 48 h post-marathon..... 123

Annexure B:

Figure B-1: A summary of the unfolded PCA of the beetroot and placebo interventions over time 128

Figure B-2: Principal component analysis plots representing the inter-cohort differentiation of (a) pre-marathon, (b) post-marathon, (c) 24 h post-marathon, and 48 h post-marathon metabolic profiles 128

LIST OF ABBREVIATIONS

<i>Abbreviation</i>	<i>Meaning</i>	<i>Abbreviation</i>	<i>Meaning</i>
8-OH-dG	8-Hydroxy-2-deoxyguanosine	IMA	Ischemia-modified albumin
AA	Amino acids	K ⁺	Potassium
AGE	Advanced glycation end-products	LC	Liquid chromatography
ALT	Alanine aminotransferase	LCFA	Long-chain fatty acids
AMP	Adenosine monophosphate	Mb	Myoglobin
ANOVA	Analysis of variance	MCH	Mean corpuscular haemoglobin volume
ASCA	ANOVA simultaneous component analysis	MCHC	Mean corpus haemoglobin concentration
AST	Aspartate aminotransferase	MCV	Mean corpus volume
ATP	Adenosine triphosphate	Mg ²⁺	Magnesium
BCAA	Branched-chain amino acid	MMP-3	Metalloproteinase-3
BNP	Brain natriuretic peptide	MS	Mass spectrometry
BUN	Blood-urea-nitrogen	Ms	Mass spectra
Ca ²⁺	Calcium	mTORC 1	Mammalian target of rapamycin complex 1
ccCK	Caspase-cleaved cytokeratin	Na ⁺	Sodium
CK	Creatine kinase	NAD ⁺	Nicotinamide adenine dinucleotide
CK-MB	Creatine kinase-myocardial band	NO	Nitric oxide
Cl ⁻	Chloride	NSAID	Non-steroidal inflammatory drugs
Co-A	Coenzyme A	NWU	North-West University
COMP	Cartilage oligomeric matrix protein	NT	Neurotransmitter
CPT	Carnitine palmitoyl transferase	NT-proBNP	N-terminal pro-brain natriuretic peptide
CRP	C-reactive protein	OCFA	Odd-chain fatty acid
cTnI	Cardiac troponin I	OWE	Open window effect

<i>cTnT</i>	<i>Cardiac troponin T</i>	<i>P1NP</i>	<i>Pro-collagen type I N-terminal pro-peptide</i>
<i>CV</i>	<i>Coefficient of variation</i>	<i>P³⁻</i>	<i>Phosphate</i>
<i>CXT</i>	<i>Cross-linked C-telopeptide of type I collagen</i>	<i>PCA</i>	<i>Principal component analysis</i>
<i>DNA</i>	<i>Deoxyribonucleic acid</i>	<i>Ph.D.</i>	<i>Philosophiae Doctor</i>
<i>EIH</i>	<i>Exercise-induced hypertension</i>	<i>PL</i>	<i>Platelets</i>
<i>ER</i>	<i>Endurance running</i>	<i>PLS-DA</i>	<i>Partial least squares – discriminant analysis</i>
<i>esRAGE</i>	<i>Endogenous secretory receptor of advanced glycation end-products</i>	<i>QC</i>	<i>Quality control</i>
<i>ETC</i>	<i>Electron transport chain</i>	<i>RAGE</i>	<i>Receptor of advanced glycation end-products</i>
<i>FA</i>	<i>Fatty acid</i>	<i>RANKL</i>	<i>Receptor activator of nuclear factor κb ligand</i>
<i>FAD⁺</i>	<i>Flavin adenine nucleotide</i>	<i>RBC</i>	<i>Red blood cell</i>
<i>FDR</i>	<i>False discovery rate</i>	<i>ROS</i>	<i>Reactive oxygen species</i>
<i>GC</i>	<i>Gas chromatography</i>	<i>RNA</i>	<i>Ribonucleic acid</i>
<i>GCxGC-TOFMS</i>	<i>Two-dimensional gas chromatography time-of-flight mass spectrometry</i>	<i>TAG</i>	<i>Triacylglycerol</i>
<i>GM</i>	<i>Gut microbiome</i>	<i>TBARS</i>	<i>Thiobarbituric acid reactive substances</i>
<i>GTP</i>	<i>Guanosine triphosphate</i>	<i>TCA</i>	<i>Tricarboxylic acid</i>
<i>Hb</i>	<i>Haemoglobin</i>	<i>TIM</i>	<i>Tissue injury model</i>
<i>Hct</i>	<i>Haematocrit</i>	<i>TNF-α</i>	<i>Tumour necrosis factor alpha</i>
<i>H-FABP</i>	<i>Heart-type fatty acid-binding protein</i>	<i>TP</i>	<i>Total protein</i>
<i>HSP</i>	<i>Heat shock protein</i>	<i>VO_{2max}</i>	<i>Maximum volume of oxygen</i>
<i>IFN-γ</i>	<i>Interferon-gamma</i>	<i>WBC</i>	<i>White blood cells/leukocytes</i>
<i>Ig</i>	<i>Immunoglobulin</i>	<i>W_{max}</i>	<i>Maximal work capacity</i>
<i>IL</i>	<i>Interleukin</i>	<i>YKL-40</i>	<i>Chitinase 3-like protein</i>

CHAPTER 1: PREFACE

1.1. BACKGROUND AND MOTIVATION

While moderate-intensity physical activity poses an almost endless list of benefits to human health, endurance running (ER; i.e. ER races) reportedly provokes various potentially deleterious physiological and biochemical effects, that range from cardiovascular dysfunction (Christensen *et al.*, 2017), and structural damage (Da Ponte *et al.*, 2018), to severe inflammation (Rubio-Arias *et al.*, 2019) and susceptibility to upper respiratory tract infections (Robson-Ansley *et al.*, 2012). Although these effects (amongst others) are relatively well-characterised, a current void exists in terms of a holistic representation of the metabolic adaptations induced by endurance races and how this relates to the previously described physiological and immunological effects. This is most likely due to the fact that previous investigations either: (1) employed targeted or semi-targeted approaches (Lewis *et al.*, 2010; Schader *et al.*, 2020), focusing only on specific metabolic fluctuations; (2) were based on findings obtained by laboratory approaches to simulating endurance runs (i.e. treadmill runs) (Davison *et al.*, 2018; Howe *et al.*, 2018), which may exclude certain confounders and therefore reduce the relatability of these findings to the true consequential nature of endurance races and the recovery thereof; and/or (3) omit to interlink these metabolic fluctuations with each other, subsequently failing to provide an explanation for these fluctuations in context of the study. Depending on the duration and intensity of ER, the most prominent metabolic adaptations induced by these races reportedly persist for approximately 1–72 h (Nieman *et al.*, 2013; Daskalaki *et al.*, 2015; Davison *et al.*, 2018; Nieman *et al.*, 2019), while physiological and immunological effects may last for a few days or even weeks (Langberg *et al.*, 2000; De Oliveira Assumpção *et al.*, 2013; Ramos-Campo *et al.*, 2016).

Regardless of the possible detrimental nature of some of these effects, ER races remain a popular global competitive pass-time and leisure activity, which has subsequently provided a platform for the commercialisation of various recovery modalities and strategies. Some of the modalities that have been investigated for the purpose of improving performance and/or expediting physiological recovery succeeding endurance exercise include heat- and cryotherapy, massage therapy, compression garments, and non-steroidal anti-inflammatory drugs (Howatson & Van Someren, 2008; Brown *et al.*, 2017; Wilson *et al.*, 2018; Peake, 2019). However, since the repeated utilisation of some of these modalities may be costly and in some instances require extensive physiological knowledge, more sustainable, cost-effective supplementation strategies, such as the ingestion of functional foods (cherries, pomegranates, blueberries, beetroot, green leafy vegetables, etc.) have gained some scientific attention (Howatson *et al.*, 2010; McLeay *et al.*, 2012; Clifford *et al.*, 2017b; Hurst *et al.*, 2019). The rationale behind the use of these foods is mostly based on their high phytonutrient content, which may provide additional advantages besides its calorie-content (Nahar *et al.*, 2020). Beetroot, in particular, has potent anti-inflammatory, vasodilatory, and antioxidant properties (Clifford *et al.*, 2017a), as well as insulin and glucose regulating abilities (Mirmiran *et al.*, 2020) that reportedly enhance athlete

performance (Australian Institute of Sport, 2019). However, whether the properties of functional foods and beetroot, in particular, may be applicable in terms of the metabolic recovery of athletes after ER, remains to be elucidated.

In an attempt to address the aforementioned scientific voids, an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach was employed to further elucidate the current understanding of exercise-induced metabolic changes and the recovery thereof, and to determine the effectiveness of beetroot juice as a metabolic recovery supplement. Withal, a better understanding of these may not only provide clues to more efficient recovery and performance-enhancing strategies, but also aid in identifying the effective dose-dependent ingestion of beetroot juice as a recovery supplement.

1.2. AIMS AND OBJECTIVES

1.2.1. Aims

This investigation aimed to use an untargeted GCxGC-TOFMS-based serum metabolomics research approach to:

1. Elucidate the holistic effects of a marathon on the serum metabolome of endurance athletes.
2. Investigate the unaided metabolic recovery trend of athletes within 48 h post-marathon.
3. Determine whether or not, the ingestion of beetroot juice provides an added advantage towards the metabolic recovery of athletes within 48 h post-marathon.

1.2.2. Objectives

These aims will be accomplished by completing the following objectives:

1. Differentiate between the pre- and immediately post-marathon serum metabolite profiles of all athletes (n=31) to determine the effects of this perturbation on the human metabolome.
2. Elucidate the unaided metabolic recovery trend of athletes within 48 h post-marathon by comparing the recovery metabolic profiles (24 h and 48 h post-marathon) of a sub-section of these marathon athletes (n=16) who received placebo supplements to the immediate post-marathon serum metabolite profiles of the same athletes (prior to placebo ingestion), while using pre-marathon profiles as a baseline (recovery) reference.
3. Compare the recovery metabolic profiles (24 h and 48 h post-marathon) of the remaining sub-section of marathon athletes (n=15) who received beetroot juice supplements after the marathon, to the

corresponding metabolic profiles of the placebo-ingesting cohort, as a means of evaluating the efficacy of beetroot juice as a post-marathon metabolic recovery supplement.

1.2.3. Supporting objectives

In addition to the aforementioned objectives, the following supporting objectives will be employed using the resulting data:

1. Confirm the validity of the results obtained by means of quality assurance procedures, using the quality control samples.
2. Apply various multivariate and univariate statistical analysis to identify the metabolite markers pertinent to the respective aims of this investigation.
3. Interpret these significant metabolic adaptations based on previously published literature.

1.3. STRUCTURE OF THESIS AND RESEARCH OUTPUTS

This thesis is constructed to comply with the specific requirements of the North-West University (NWU), for the completion of a Philosophiae Doctor (Ph.D.) degree (in Biochemistry) in article format. As such, this thesis includes an acknowledgements section, followed by a summary of the current investigation and a list of figures, tables and abbreviations. The succeeding chapters consist of an introduction, methods and materials, results, discussion, and conclusion sections, followed by a list of references used.

In summary, **Chapter 1** consists of the preface which gives a brief background and motivation of the scientific relevance of this study, as well as the aims and objectives, structure of the thesis, and the contributions made by all co-authors, co-workers and collaborators.

Hereafter, **Chapter 2** provides a detailed overview of the literature relevant to the research topic, as a basis for understanding the related metabolism. A part of this chapter has been submitted for publication as a review paper (see Annexure C):

- Stander, Z., Luies, L. & Loots, D.T. 2020. The acute systematic biochemical adaptations induced by endurance running. Submitted for publication to *Biological Reviews* (Manuscript number: BRV-04-2020-0089). Impact factor: 10.28

Chapter 3 describes the general experimental design, including sample collection and the research methodology (i.e. metabolite extraction, analysis, and data handling) used during this investigation, as well as participant characteristics, selection and ethical approval.

In **Chapter 4**, the aforementioned methodology was used to analyse and differentiate the pre- and immediately post-marathon serum metabolite profiles of the athletes (n=31). The significant metabolite markers identified via statistical selection, pertaining to the metabolic variation between these profiles, were interpreted in light of their role in known metabolic pathways, subsequently elucidating the holistic effects of a marathon on the metabolome of the athletes. This chapter has already been published as an original research paper (see Annexure C):

- Stander, Z., Luies, L., Mienie, L.J., Keane, K.M., Howatson, G., Clifford, T., Stevenson, E.J. & Loots, D.T. 2018. The altered human serum metabolome induced by a marathon. *Metabolomics*, 14(150). Impact factor: 3.16

Chapter 5 investigated the unaided metabolic recovery trend over time-frame of 48 h post-marathon, by comparing the 24 h and 48 h post-marathon metabolic profiles of the athletes (n=16) whom incrementally ingested placebo supplements after completion of the marathon, to the corresponding immediately post-marathon serum metabolite profiles of each individual. These results provided insight into the ability of the human body to recover unassisted or “naturally” within 48 h post-race. This chapter has already been published as an original research paper (see Annexure C):

- Stander, Z., Luies, L., Mienie, L.J., Van Reenen, M., Howatson, G., Keane, K.M., Clifford, T., Stevenson, E.J. & Loots, D.T. 2020. The unaided recovery of marathon-induced serum metabolome alterations. *Scientific Reports*, 10(11060). Impact factor: 4.52

Chapter 6 details the metabolic comparison of the recovery profiles (24 h and 48 h post-marathon) of the remaining subset of athletes (n=15) that ingested beetroot juice supplements after the marathon, to that of the placebo cohort over the same period of time. These results provided insight into the effectivity of beetroot juice supplementation within 48 h post-marathon as a possible metabolic recovery agent. This chapter has been drafted for publication as an original research paper (see Annexure C):

- Stander, Z., Luies, L., Van Reenen, M., Howatson, G., Keane, K.M., Clifford, T., Stevenson, E.J. & Loots, D.T. 2020. Beetroot juice – a suitable post-marathon metabolic recovery supplement? To be submitted to *International Journal of Behavioural Nutrition and Physical Activity*. Impact factor: 5.54

Lastly, **Chapter 7** provides an all-inclusive conclusion, bringing into context all respective aims of this investigation, and also briefly discusses possible future prospects emanating from the results.

1.4. AUTHOR CONTRIBUTIONS

Although Zinandr  Stander is the primary author and investigator of this thesis, additional contributions made by all co-authors, colleagues, and collaborators are disclosed in Table 1-1.

Table 1-1: *Academic contributions of all parties involved in this thesis.*

Relevant party	Academic role	Contribution
Mrs Zinandr� Stander (B.Sc. Hons Biochemistry)	Ph.D. student	Primary investigator and author: Conceptualisation of the metabolomics investigation of the involved samples, drafting of thesis and manuscripts, data analysis, and interpretation. The first author of all the academic outputs emanating from this Ph.D.
Prof Du Toit Loots (Ph.D. Biochemistry)	Supervisor	Supervised and coordinated all aspects of the study including student guidance, metabolomics study conceptualisation, data analysis, interpretation, proofing of thesis and manuscripts. Co-author of all the academic outputs emanating from this Ph.D.
Dr Laneke Luies (Ph.D. Biochemistry)	Co-supervisor	Supervision and guidance of the student, data analysis, interpretation, drafting and in-depth proofing of thesis and manuscripts. Co-author of all the academic outputs emanating from this Ph.D.
Mrs Derylize Beukes (B.Sc. Hons Biochemistry)	Colleague	Responsible for sample analysis and the training of Mrs. Stander as part of her duties as Laboratory Manager.
Dr Mari van Reenen (Ph.D. Biochemistry)	Co-author	Assisted with all data processing and statistical analysis of this study, as well as the proofing of manuscripts. Co-author to two of the four academic outputs.
Prof Japie Mienie (Ph.D. Biochemistry)	Co-author	Assisted in data analysis, interpretation and proofing of relevant manuscripts. Co-author to two of the four academic outputs.
Prof Glyn Howatson (Ph.D. Exercise Physiology)		
Prof Emma Stevenson (Ph.D. Sport and Exercise Nutrition)		
Dr Tom Clifford (Ph.D. Exercise and Health Nutrition)	International collaborators and co-authors	Responsible for sample collection, study conceptualisation, manuscript proofing. Co-authors of all the original data-generated outputs emanating from this Ph.D.
Dr Karen Keane (Ph.D. Sports Science)		

The following statement from the study supervisors and primary author confirm their respective roles in this study and give permission that the data generated, and conclusions made, may form part of this thesis:

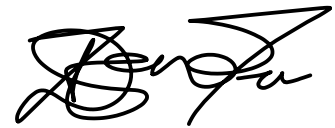
I declare that my role in this study, as indicated in Table 1-1, is an accurate representation of my actual contribution, and I hereby give my consent that this work may be published as part of the Ph.D. thesis of Zinandré Stander.



Prof Du Toit Loots
(Supervisor)



Dr Laneke Luies
(Co-supervisor)



Mrs Zinandré Stander
(Ph.D. student)

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CHAPTER 2: LITERATURE REVIEW

Parts of this chapter have been submitted for publication:

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2.1. INTRODUCTION

ER races is a highly competitive, well-established sport, that is categorised as either a long-distance race (>5 km) (Lieberman *et al.*, 2009), half-marathon (21 km), marathon (42 km) or ultra-marathon (>42 km) (Kruger & Saayman, 2013). Participation in these races has increased exponentially over the past decade, with participant rationales ranging from health-related benefits to intrinsic rewards and camaraderie (Kruger & Saayman, 2013). Running these distances require extensive mental and physical conditioning along with carefully planned personalised diets and extreme mental preparation. One of the most crucial factors adversely influencing athlete performance during an endurance run is fatigue (Rapoport, 2010). Athlete performance may be influenced by a number of factors, including: (1) the depletion of fuel substrates, (2) the intensity and duration of muscle contractions, (3) maximum volume of oxygen (VO_{2max}) intake and (4) nutritional support during training or the race (Rapoport, 2010). Although many of these factors are controllable to some extent, numerous predictive factors are environmental (Oliveira *et al.*, 2017) and/or genetic (Grealy *et al.*, 2015) and are therefore difficult or impossible to control for example; forefoot bone length (Ueno *et al.*, 2018), ageing (Connick *et al.*, 2015; Van Beek *et al.*, 2016), epigenetics (Ehlert *et al.*, 2013), and a vast array of DNA polymorphisms that are determinants of individual endurance, cardiovascular fitness, mitochondrial function, psychosomatic skills and VO_{2max} , etc. (Ahmetov & Fedotovskaya, 2012; Sarzynski *et al.*, 2017; Znazen *et al.*, 2017). Nevertheless, moderate physical activity is mainly considered beneficial to human health (Rowe *et al.*, 2014), especially with regards to body-weight regulation (Vaynman & Gomez-Pinilla, 2006; Venables & Jeukendrup, 2008), neurological stimulation and subsequent endorphin production (Dishman & O'Connor, 2009). On the other hand, ER has also been associated with various adverse effects, including severe mechanical shearing, tendon and nerve damage (Bonasia *et al.*, 2015), a compromised immunity, and the propensity to various acute/chronic pathologies. Cardiovascular dysfunction (Carbone *et al.*, 2017), pulmonary arterial hypertension (La Gerche *et al.*, 2014), upper respiratory infection (Robson-Ansley *et al.*, 2012), as well as sudden cardiac arrest (Webner *et al.*, 2012; Harmon *et al.*, 2016), are some of the pathological risk-factors associated with ER.

Besides these phenotypical changes, ER also has a profound biochemical effect on athletes. A comprehensive understanding of the latter provides clues to demographic-specific performance optimisation methods as well as improved systematic damage prevention strategies. Here, we discuss the previously identified biomarkers

adaptations induced by ER (summarised in Table 2-1 and Table 2-2) and discuss these in terms of their associated pathologies, internal damage and pathway complexity. Furthermore, since a standard means of classifying ER athletes according to experience/performance is currently lacking in literature, we suggest a proficiency classification system (Table 2-3). Lastly, a brief discussion regarding the unaided and functional food supplemented recovery of athletes after ER is provided.

2.2. PHYSIOLOGICAL (STRUCTURAL) AND IMMUNOLOGICAL BIOMARKER ADAPTATIONS

2.2.1. Blood biochemistry

The majority of the research investigating the biological effects of ER is based on the utilisation of blood samples (either the plasma and/or serum) as the preferred medium. Since blood serves as the primary transporting mechanism for all molecules excreted or produced in response to physiological and pathological stimuli (Psychogios *et al.*, 2011), it is considered a popular medium for immunological and metabolic investigations. Commonly measured blood parameters include platelets (PL), haematocrit (Hct), mean corpus haemoglobin concentration (MCHC), mean corpuscular volume (MCV), mean corpuscular haemoglobin volume (MCH), red blood cells (RBC, also called erythrocytes) and haemoglobin (Hb). Although the majority of the literature investigating the changes induced by ER, reported only minor and/or largely non-significant changes to these parameters after ER events (Table 2-1), a selected few observed significant fluctuations (Table 2-1). Kratz *et al.* (2006) investigated the effect of marathon running on various platelet-activation biomarkers in 32 healthy athletes participating in the Boston Marathon and observed elevated RBC, PL counts, Hct, Hb and MCHC. These elevations are supported by a few studies (Table 2-1) and are mainly ascribed to an acute-phase inflammatory response due to possible tissue damage, and/or dehydration (Kratz *et al.*, 2006; Widmaier *et al.*, 2016). Although Da Ponte *et al.* (2018) also observed elevated PL and MCHC in 22 Italian males participating in an uphill marathon, significant reductions in Hct were observed regardless of the mildly dehydrated state of the athletes. This coincides with the findings of Liu *et al.* (2018), in which a reduction in Hct was accompanied by reductions in RBC and elevations in haptoglobin, reticulocytes (immature RBC), and ferritin, in 19 male athletes participating in a 24 h ultra-marathon. Lui *et al.* (2018), attributed this observation to haemolysis that may be classified as “sports-anaemia” (Wang *et al.*, 2010; Chiu *et al.*, 2015). The aforementioned haemolysis may be caused by a continuous mechanical force (foot-strike, rhabdomyolysis, etc.) shearing and the subsequent release of RBC content (Lippi & Sanchis-Gomar, 2019), and hence the reductions in the RBC, Hct, and haem (Robach *et al.*, 2014), accompanied by elevations of ferritin and iron. Elevated blood ferritin levels have also been associated with hepatic damage, poor iron status, and angiogenesis. Elevated levels of haptoglobin are crucial during haemolysis, as it is thought to react with Hb to prevent the oxidative capabilities of RBC components (Robach *et al.*, 2014; Chiu *et al.*, 2015; Lippi & Sanchis-Gomar, 2019). This is counteracted by the activation of erythropoiesis, leading to the elevations in the reticulocytes observed (Table 2-1).

Table 2-1: ER-induced physiological and immunological-associated biomarker adaptations reported in recent (2000-2020) literature (arranged according to publication year).

Reference	Exercise type	Cohort	Sampling media	Physiological and immunological adaptations when compared to baseline measurements	
Kratz <i>et al.</i> (2002)	Marathon	37 participants (32 male; 5 female)	Blood; before, within 4 h after and 24 h ⁺ post-race	Within 4 h post-race: Blood biochemistry: RBC (↓) ^a ; Hct (↓) ^a ; Hb (↑) ^a ; PL (↑) ^a ; Alb (↑) ^a ; TP (↑) ^a ; MCV (↓) ^a ; MCH (↑) ^a ; MCHC (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; ALT (↑) ^a ; AST (↑) ^a ; Bilirubin (↑) ^a ; ALP (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; cTnI (↑) ^a ; Mb (↑) ^a Renal damage: BUN (↑) ^a Electrolyte markers: Na ⁺ (↓)*; K ⁺ (↓)*; Cl ⁻ (↓) ^a ; Mg ²⁺ (↓) ^a ; Ca ²⁺ (↑) ^a ; P ³⁻ (↑) ^a Immune response: WBC (↑) ^a ; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Basophils (↑) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ; Globulin (↓) ^a	
Niessner <i>et al.</i> (2003)	Marathon	19 participants (17 male; 2 female)	Blood; before and immediately after the race	Post-race: Cardiac muscle damage: Pro-ANP (↑) ^a ; NT-proBNP (↑)* Hormonal response: Aldosterone (↑) ^a	
Uchakin <i>et al.</i> (2003)	Marathon (White Rock and Cowton marathons)	15 male participants	Blood; before, 20 min and 60 min after the race as well as 2 ⁺ , 5 ⁺ and 8-days ⁺ post-race	Within 20 min post-race: Blood biochemistry: Hct (↑)*; Hb (↑)* Muscle and hepatic damage: CK (↑) ^a Immune response: Granulocytes (↑) ^a ; IL-6 (↑) ^a ; TNF-α (↑) ^a ; Lymphocytes (↓)*; IL-1β (-) LPS stimulated: IL-2 (↓) ^a ; IL-1β (↓)*; IFN-γ (↓) ^a ; IL-10 (↓)*; TNF-α (↓) ^a ; IL-6 (↓)* Hormonal response: ACTH (↑) ^a ; β-endorphin (↑) ^a ; Growth hormone (↑) ^a ; Cortisol (□) ^a	Within 60 min post-race: Blood biochemistry: Hct (↑)* ^{a,c} ; Hb (↑)* ^{a,c} Muscle and hepatic damage: CK (↑) ^{a,b} Immune response: Granulocytes (↓) ^{a,c} ; IL-6 (↑) ^{a,c} ; TNF-α (↑) ^a ; Lymphocytes (↓) ^{a,c} ; IL-1β (-) LPS stimulated: IL-2 (↓) ^{a,c} ; IL-1β (↓)* ^{a,b} ; IFN-γ (↓) ^{a,c} ; IL-10 (↓)* ^{a,b} ; TNF-α (↓) ^{a,b} ; IL-6 (↓)* ^{a,b} Hormonal response: ACTH (↑) ^{a,c} ; β-endorphin (↑) ^{a,c} ; Growth hormone (↑) ^{a,c} ; Cortisol (□) ^{a,c}
Goudie <i>et al.</i> (2006)	Marathon	14 participants (<i>genders not provided</i>)	Blood; patients admitted to St Thomas hospital post-race	Post-race: Electrolyte markers: Na ⁺ (↓) ^a	
Kratz <i>et al.</i> (2006)	Marathon	32 participants (27 male; 5 female)	Blood; before and immediately after the race	Post-race: Blood biochemistry: RBC (↓) ^a ; Hct (↑) ^a ; Hb (↑) ^a ; PL (↑)*; MCV (↓) ^a ; MCH (↑)*; MCHC (↑) ^a ; Reticulocyte (↑) ^a Immune response: WBC (↑) ^a ; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Basophils (↓) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ;	
Leers <i>et al.</i> (2006)	Marathon	27 participants (25 male; 2 female)	Blood; before, immediately after and 24 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑) ^a Electrolyte markers: Na ⁺ (↓)*; Cl ⁻ (↓) ^a ; K ⁺ (-)	

Kim <i>et al.</i> (2007)	Ultra-marathon (200 km)	54 male participants	Blood; before, after 100 km and immediately post-race	100 km of race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: Hs-CRP (↑) ^a ; IL-6 (↑) ^a ; TNF-α (↑)* Cartilage damage: COMP (↑) ^a Blood biochemistry: Hct (↑)*	Post-race: Muscle and hepatic damage: CK (↑) ^{a,d} ; LDH (↑) ^{a,d} ; AST (↑) ^{a,d} ; ALT (↑) ^a Immune response: Hs-CRP (↑) ^{a,d} ; IL-6 (↑) ^{a,d} ; TNF-α (↑)* Cartilage damage: COMP (↑) ^{a,d} Blood biochemistry: Hct (↑) ^{*,b}
Mouzopoulos <i>et al.</i> (2007)	Ultra-marathon (245 km)	16 male participants	Blood; 5-days before, immediately after as well as 1 ⁺ , 3 ⁺ and 5-days ⁺ post-race	Post-race: Bone biochemistry: ICTP (↑)*; PICP (↓) ^a ; Osteocalcin (↓) ^a ; ALP (↓) ^a Hormonal response: Parathyroid hormone (↑) ^a Electrolyte markers: Ca ⁺ (↓)*	
Siegel <i>et al.</i> (2007)	Marathon	33 participants (<i>sex not provided</i>)	Blood; before and after the race	Post-race: Immune response: IL-6 (↑) ^a ; CRP (–) Muscle and hepatic damage: CK (↑) ^a Hormonal response: Prolactin (↑) ^a ; Arginine vasopressin (↑) ^a Renal damage: BUN (↑) ^a	
Lippi <i>et al.</i> (2008)	Half-marathon	15 male participants	Blood; before, immediately after, 3 h, ++	Post-race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; Mb (↑) ^a ; cTnT (–)	
Knechtle <i>et al.</i> (2009)	Ultra-marathon (100 km)	39 male participants	Blood and urine; before and immediately after	Post-race: Blood biochemistry: Hct (↓) ^a Electrolyte markers: Na ⁺ (↓) ^a	
Kersch-Schindl <i>et al.</i> (2009)	Ultra-marathon (246 km)	18 participants (16 male; 2 female)	Blood; before, within 15 min after as well as 3-days ⁺ post-race	Post-race: Bone biochemistry: CTX (↑) ^a , Osteocalcin (↓) ^a , Osteoprotegerin (↑) ^a , RANKL (↑)*	
Fu <i>et al.</i> (2010)	Half-marathon	17 adolescent male participants	Blood; before, within 12 min after as well as 4 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a , NT-proBNP (↑) ^a	
Burge <i>et al.</i> (2011)	Ultra-marathon (100 km)	50 male participants	Blood and urine; before and after the race	Post-race: Blood biochemistry: Hct (↓)*; Hb (↓)* Electrolyte markers: Na ⁺ (↑) ^a , K ⁺ (↑) ^a Hormonal response: Aldosterone (↑) ^a , Copeptin (↑) ^a	
Kipps <i>et al.</i> (2011)	Marathon	88 participants (53 male; 35 female)	Blood; before and after the race	Post-race: Electrolyte markers: Na ⁺ (↓) ^a	

Knechtle <i>et al.</i> (2011)	Ultra-marathon (100 km)	145 male participants	Blood; before and immediately after the race	Post-race Blood biochemistry: Hct (↓) ^a ; Hb (↓) ^a Electrolyte markers: Na ⁺ (↓)*		
Lippi <i>et al.</i> (2011)	Half-marathon	15 male participants	Blood; pre-fasting, 48 h after last training session, immediately after the race as well as 3 h ⁺ , 6 h ⁺ and 24 h ⁺ post-race	Post-race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; GGT (↑) ^a ; Bilirubin (↑) ^a ; ALT (–); ALP (–)		
McCullough <i>et al.</i> (2011)	Marathon	25 participants (12 male; 13 female)	Blood: before, after and 24 h ⁺ post-race	Within 4 h post-race: Blood biochemistry: Alb (↑) ^a ; TP (↑) ^a ; MCV (↓) ^a ; MCHC (↑) ^a ; MCH (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; ALT (↑) ^a ; AST (↑) ^a ; Bilirubin (↑) ^a ; ALP (↑) ^a ; Aldolase (↑) ^a , ALP (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; cTnI (↑) ^a ; BNP (↑) ^a Renal damage: BUN (↑) ^a ; Cystatin (↑)*; NGAL (↑) ^a ; KIM-1 (↑) ^a Electrolyte markers: Na ⁺ (↑)*; K ⁺ (↑) ^a Immune response: Globulin (–)		
La Gerche <i>et al.</i> (2012)	Marathon	9 cyclists ⁺ , 13 triathlon- ⁺ and 7 marathon participants	Blood; 2–3 weeks before, immediately after and 6–11 days ⁺ post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; BNP (↑) ^a (<i>correlated with cardio-graphic tests</i>) ⁺		
Waśkiewicz <i>et al.</i> (2012)	Ultra-marathon (±168.5 km; 24 h)	19 male participants	Blood; before, at marathon distance, within 12 h and immediately after the ultra-marathon	Marathon length: Blood biochemistry: RBC (↑)*; Hb (↑)*; Hct (↑)*; PL (↑)*; MCV (↓)*; CO ₂ (↓)*; O ₂ (↓)* Muscle and hepatic damage: CK (↑) ^a ; AST (–); ALT (–); GGT (–) Electrolyte markers: Na ⁺ (↑)*; K ⁺ (↑) ^a ; Ca ²⁺ (↓)* Immune response: WBC (↑) ^a ; Lymphocytes (↑)*; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Eosinophils (↓)*; Basophils (↑)*; IL-6 (↑) ^a , CRP (–);	12 h of running: Blood biochemistry: RBC (↑)* ^{a,c} ; Hb (↑)* ^{a,c} ; PL (↑)* ^{a,c} ; O ₂ (↑)* ^{a,b} ; Hct (↓)* ^{a,c} ; MCV (↓)*; CO ₂ (↓) ^{a,c} Muscle and hepatic damage: CK (↑) ^{a,b} ; AST (–); ALT (–); GGT (–) Electrolyte markers: Na ⁺ (↑)* ^{a,b} ; K ⁺ (↑) ^{a,b} ; Ca ²⁺ (↓) ^a Immune response: WBC (↑) ^{a,c} ; Lymphocytes (↓)* ^{a,c} ; Monocytes (↑) ^{a,b} ; Neutrophils (↑) ^{a,b} ; Eosinophils (↓) ^{a,c} ; Basophils (↑)* ^{a,c} ; IL-6 (↑) ^{a,b} , CRP (↑) ^a	Post-race: Blood biochemistry: RBC (↑)* ^{a,c} ; Hb (↑)* ^{a,c} ; PL (↑)* ^{a,c} ; O ₂ (↑) ^{a,b} ; Hct (↓)* ^{a,c} ; MCV (↓)*; CO ₂ (↓) ^{a,c} Muscle and hepatic damage: CK (↑) ^{a,b} ; AST (↑) ^a ; ALT (↑) ^a ; GGT (↑)* Electrolyte markers: Na ⁺ (↓)* ^{a,c} ; K ⁺ (↑) ^{a,b} ; Ca ²⁺ (↑)* ^{a,b} Immune response: WBC (↑) ^{a,b} ; Lymphocytes (↓)* ^{a,b} ; Monocytes (↑) ^{a,b} ; Neutrophils (↑) ^{a,c} ; Eosinophils (↓) ^{a,b} ; Basophils (↑) ^{a,-} ; IL-6 (↑) ^{a,b} , CRP (↑) ^{a,b}

Costa <i>et al.</i> (2013)	Multi-stage-marathon (225 km)	74 participants (46 male; 28 female) and 12 controls ⁺ (5 male; 7 female)	Blood; before and after each stage	Stages 1-5: Electrolyte markers: Na ⁺ (↓)* (<i>hyponatremia in eight athletes</i>)		
Klapcinska <i>et al.</i> (2013)	Ultra-marathon (48 h; 183–320 km)	7 male participants	Blood; before, after running 12 h, 24 h and within 10 min after the race as well as 24 h ⁺ and 28 h ⁺ post-race	12 h of running: Blood biochemistry: RBC (↑)*; Hct (–); Hb (↑)*; PL (↑) ^a ; MCV (↓) ^a ; MCH (↑)*; MCHC (↑) ^a ; pCO ₂ (↓) ^a Electrolyte markers: Na ⁺ (↓)*; K ⁺ (↑) ^a ; Ca ²⁺ (↓)* Iron panel: Ferritin (↑) ^a ; Iron (□)* Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; Lymphocytes (↑)*	24 h of running: Blood biochemistry: RBC (–) ^c ; Hct (↓)* ^c ; Hb (↑)* ^c ; PL (↑) ^{a,c} ; MCV (↓) ^{a,c} ; MCH (↑)*; MCHC (↑) ^{a,b} ; pCO ₂ (↓) ^{a,b} Electrolyte markers: Na ⁺ (↓)* ^c ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) ^{a,c} Iron panel: Ferritin (↑) ^{a,b} ; Iron (↓)* ^b Immune response: WBC (↑) ^{a,c} ; Neutrophils (↑) ^{a,c} ; Monocytes (↑) ^{a,c} ; Lymphocytes (↑)*	Post-race: Blood biochemistry: RBC (↓)*; Hct (↓) ^a ; Hb (↑)*; PL (↑)* ^c ; MCV (↓) ^{a,b} ; MCH (↑)*; MCHC (↑) ^{a,c} ; pCO ₂ (↓) ^{a,c} Electrolyte markers: Na ⁺ (↓) ^{a,c} ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) ^{a,c} Iron panel: Ferritin (↑) ^{a,b} ; Iron (□)* ^c Immune response: WBC (↑) ^{a,c} ; Neutrophils (↑) ^{a,c} ; Monocytes (↑) ^{a,c} ; Lymphocytes (↑)* ^c
Robach <i>et al.</i> (2014)	Ultra-marathon (166 km)	22 male participants	Blood, before and within 20 min after the race as well as 2 ⁺ , 5 ⁺ , 9 ⁺ and 16 ⁺ – days post-race	Post-race: Blood biochemistry: Hb (↑)*, Haptoglobin (↓) ^a Cardiac muscle damage: CK-MB (↑) ^a ; Mb (↑) ^a ; cTnT (–) Muscle and hepatic damage: LDH (↑) ^a ; Bilirubin (↑)* Immune response: WBC (↑) ^a , CRP (↑) ^a , Orosomucoid (↑) ^a		
Salvagno <i>et al.</i> (2014)	Ultra-marathon (60 km)	18 participants (<i>sex not provided</i>)	Blood; before, within 10 min after as well as 1 h ⁺ after	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a ; Galectin-3 (↑) ^a		
Vuolteenaho <i>et al.</i> (2014)	Marathon	46 male participants	Blood; before and immediately after	Post-race: Cartilage damage: COMP (–); MMP (↑) ^a ; YKL (↑) ^a Hormonal response: Adiponectin (↑) ^a ; Leptin (↓)*; Resistin (↑) ^a		
Hewing <i>et al.</i> (2015)	Marathon	167 participants (78 male; 89 female)	Blood; before, immediately after as well as 2 weeks ⁺ post-race	Post-race: Blood biochemistry: Hb (↑) ^a ; Hct (↑) ^a ; TP (↑) ^a Electrolyte markers: Na ⁺ (↑) ^a Cardiac muscle damage: cTnT (↑) ^a ; NT-proBNP (↑) ^a Renal damage: Cystatin C (↑) ^a Immune response: CRP (↓) ^a		

Jastrzebski <i>et al.</i> (2015)-	Ultra-marathon (100 km)	14 male participants	Blood; before, after 25 km, 50 km, 75 km as well as immediately after the 100 km and 24 h ⁺ post-race	After 25 km: Muscle and hepatic damage: CK (↑) ^a , LDH (↑) ^a , AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a	After 50 km: Muscle and hepatic damage: CK (↑) ^a , LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a	After 75 km: Muscle and hepatic damage: CK (↑) ^a , LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a	After 100 km: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a
Khodaei <i>et al.</i> (2015)	Ultra-marathon (161 km)	20 pre-marathon participants (14 male; 6 female) and 21 post-marathon participants (17 male; 4 female)	Blood; before and immediately after	Post-race: Blood biochemistry: Hct (↓)*; Hb (↓)* Electrolyte markers: Na ⁺ (↓) ^a ; Cl ⁻ (↓) ^a ; K ⁺ (-) Renal damage: BUN (↑) ^a Cardiac muscle damage: cTnI (↑) ^a			
Kersch-Schindl <i>et al.</i> (2015)	Ultra-marathon (246 km)	19 participants (18 male; 1 female)	Blood; before, within 15 min after as well as 3-days ⁺ post-race	Post-race: Bone biochemistry: CTX (↑) ^a , Sclerostin (↓)*, Dkk-1 (↓) ^a , P1NP (↓) ^a , Cathepsin K (↓)* Muscle and hepatic damage: Myostatin (↑) ^a , Follistatin (↑) ^a			
Li <i>et al.</i> (2015)	5 km race	18 participants (9 male; 9 female)	Saliva; before and 10 min after the race	Post-race: Salivary defence (sex dependent): IgA (↑) ^a ; α-amylase (↑) ^a , TP (↑) ^a , lactoferrin (↑)*			
Suzuki <i>et al.</i> (2015)	5 h running exercise (Participant A= 34.14 km; participant B=43.05 km)	2 female participants	Blood; before, within 60 min after as well as 1 ⁺ , 3 and 5 h post-race	Participant A post-race: Blood biochemistry: Alb (↓) Hormonal response: Insulin (↑); Glucagon (↑); ACTH (↑); Vasopressin (↑)		Participant B post-race: Blood biochemistry: Alb (↑) Hormonal response: Insulin (↓); Glucagon (↑); ACTH (↑); Vasopressin (↑)	
Arakawa <i>et al.</i> (2016)	Ultra-marathon (130 km)	18 middle-aged male participants	Blood; before, after 42 km (marathon), immediately after as well as 3 ⁺ , 5 ⁺ and 7 days ⁺ post-race	Marathon length: Blood biochemistry: RBC (↑)*; Hct (↑)*; Hb (↑)*; TP (↑) ^a ; Alb (↑) ^a ; MCV (↓)*; MCH (↑)*; MCHC (↑)* Muscle and hepatic damage: CK (↑)*; LDH (↑) ^a ; AST (↑)*; ALT (↑)*; GGT (↑)*; Bilirubin (↑)* Renal damage: BUN (↑)* Hormonal response: Insulin (↓) ^a ; Leptin (↑)*; Adiponectin (↑)* Immune response: WBC (↑) ^a ; IL-6 (↑) ^a ; CRP (-); TNF-α (↑)* Iron panel: Ferritin (↑)*		Post-race: Blood biochemistry: RBC (↑)* ^c ; Hct (↑)* ^c ; Hb (↑)* ^c ; TP (↑)* ^c ; Alb (↑) ^{a,c} ; MCHC (↑)* ^c ; MCV (↓) ^{a,c} ; MCH (↓)* ^c Muscle and hepatic damage: CK (↑) ^{a,b} ; LDH (↑) ^{a,b} ; AST (↑) ^{a,b} ; ALT (↑) ^{a,b} ; GGT (↑)* ^b ; Bilirubin (↑) ^{a,b} Renal damage: BUN (↑) ^{a,b} Hormonal response: Insulin (↓) ^{a,c} ; Leptin (↓) ^{a,c} ; Adiponectin (↑)* ^b Immune response: WBC (↑) ^{a,b} ; IL-6 (↑) ^{a,c,*} ; CRP (↑) ^{a,b} ; TNF-α (↓)* ^c Iron panel: Ferritin (↑) ^{a,b} ;	

Bekos <i>et al.</i> (2016)	Marathon and half-marathon	34 marathon participants (24 male; 10 female), 36 half-marathon participants (19 male; 17 female) and 30 sedentary controls ⁺ (17 male; 13 female)	Blood and urine; before and immediately after as well as 2–7 days ⁺ post-race	Half-marathon: Blood biochemistry: RBC (↑) ^a ; Hb (↑) ^a ; PL (↑) ^a ; MCV (↑) ^a ; MCH (↑) ^a ; MCHC (↑) ^a ; Hct (–) Muscle and hepatic damage: CK (–); GGT (–) Cardiac muscle damage: CK-MB (–); sST2 (–) Electrolyte markers: Serum Na ⁺ (↑) ^a Urine fraction Na ⁺ (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; RAGE (↑) ^a ; AGE-CML (↑) ^a ; Lymphocytes (↓) ^a ; Monocytes (↓) ^a ; CRP (–); esRAGE (–); IL-1RA (–); IL-33 (–) Apoptosis: Serum HMGB1 (↑) ^a ; Serum cytokeratin 18 (↑) ^a ; Urine cytokeratin 18 (↑) ^a		Marathon: Blood biochemistry: RBC (↑) ^a ; PL (↑) ^a ; MCV (↑) ^a ; MCH (↑) ^a ; MCHC (↑) ^a ; Hct (–); Hb (–) Muscle and hepatic damage: CK (↑) ^a ; GGT (–) Cardiac muscle damage: CK-MB (↑) ^a ; sST2 (↑) ^a Electrolyte markers: Serum Na ⁺ (↑) ^a Urine fraction Na ⁺ (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; AGE-CML (↑) ^{a,d} ; Lymphocytes (↓) ^a ; Monocytes (–); CRP (–); RAGE (–); esRAGE (–); IL-1 (–); IL-33 (–) Apoptosis: Serum HMGB1 (↑) ^a ; Serum cytokeratin 18 (↑); Urine cytokeratin 18 (↑) [*]	
Jastrzebski <i>et al.</i> (2016)	Ultra-marathon (100 km)	14 male participants	Blood; before, after 20 km, 50 km, 75 km, and immediately after and 24 h ⁺ post-race	25 km of race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)*; MCHC (↓)* Electrolytes: Na ⁺ (↓)*; Ca ²⁺ (↓)*; Cl [–] (↓)*; K ⁺ (↑)* Cardiac muscle damage: Mb (↑)* Muscle and hepatic damage: Bilirubin (↑)* Immune response: WBC (↑)*	50 km of race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; MCHC (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)* Electrolytes: Na ⁺ (↓)*; Ca ²⁺ (↓)*; Cl [–] (↑)*; K ⁺ (↑)* Cardiac muscle damage: Mb (↑) ^a Muscle and hepatic damage: Bilirubin (↑)* Immune response: WBC (↑)*	75 km of race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)*; MCHC (↓)* Electrolytes: Na ⁺ (↓)*; Ca ²⁺ (↓)*; Cl [–] (↓)*; K ⁺ (↑)* Cardiac muscle damage: Mb (↑) ^a Muscle and hepatic damage: Bilirubin (↑)* Immune response: WBC (↑)	Post-race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; MCHC (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)* Electrolytes: Na ⁺ (↑)*; K ⁺ (↑)*; Ca ²⁺ (↓)*; Cl [–] (↓)* Cardiac muscle damage: Mb (↑) ^a Muscle and hepatic damage: Bilirubin (↑) ^a Immune response: WBC (↑)*
Nielsen <i>et al.</i> (2016)	Marathon and half-marathon	14 male marathon participants, 16 half-marathon participants (8 male; 8 female) and 10 male ranger cadets ⁺	Blood; before and immediately after each race	Half-marathon length: Blood biochemistry: Hct (–); Hb (–); PL (↑) ^a Immune response: WBC (↑) ^a ; IL-1β (↑)*; IL-6 (↑) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF-α (↓)*; IL-2 (–); IL-4 (–); IL-5 (–); IL-12 (–); IFN-γ (–); GM-CFS (–); VEGF (–) LPS stimulated: IL-1β (↓) ^a ; IL-6 (↓) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF (↓) ^a		Marathon length: Blood biochemistry: Hct (–); Hb (–); PL (↑) ^a Immune response: WBC (↑); IL-6 (↑) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF-α (↑)*; IL-1β (↓)*; IL-4 (↓)*; IL-12 (↓)*; VEGF (↓)*; IL-2 (–); IL-5 (–); IFN-γ (–); GM-CFS (–) No LPS stimulation of cytokines	

Shin <i>et al.</i> (2016)	Ultra-marathon (308 km)	17 marathon participants, 17 ultra-marathon (100 km) participants and 17 ultra-marathon participants (308 km) (<i>sex not provided</i>)	Blood; before, at marathon distance, after 100 km and immediately post-race	Marathon distance: Blood biochemistry: TP (↑) ^a ; Alb (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑)*; GGT (↑) ^a , ALP (↑) ^a Renal damage: BUN (↑) ^a	After 100 km: Blood biochemistry: TP (↑)*; Alb (↑)* Muscle and hepatic damage: CK (↑) ^{a,d} ; LDH (↑) ^{a,d} ; AST (↑) ^{a,d} ; ALT (↑) ^{a,d} ; Bilirubin (↑) ^a ; GGT (↓)*, ALP (↑)* Renal damage: BUN (↑) ^{a,d}	Post-race: Blood biochemistry: TP (↑) ^a , Alb (↓) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑) ^a ; GGT (↑) ^{a,d} , ALP (↓)* Renal damage: BUN (↑) ^{a,d}
Danese <i>et al.</i> (2017)	Half-marathon	30 participants (21 male; 9 female)	Blood; before and immediately after the race	Post-race: Blood biochemistry: Hct (–); Hb (–)		
Mündermann <i>et al.</i> (2017)	Marathon	45 male participants	Blood; before, after a 10-week training period ⁺ , immediately after the marathon as well as 24 h ⁺ post-race	Post-race: Cartilage damage: COMP (↑) ^a Immune response: Hs-CRP (↓) ^a , TNF-α (↓) ^a , IL-6 (↑) ^a		
Oliveira <i>et al.</i> (2017)	Marathon (hot and temperate temperatures)	26 male participants	Blood and urine, before, immediately after as well as 1 ⁺ and 3-days ⁺ after	Post-race (hot temperature): Blood biochemistry: RBC (–); Hct (↓) ^a ; Hb (↑)*; MCV (↓) ^a ; MCH (↑) ^a ; MCHC (↑) ^a , EPO (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑)*; GGT (↓)*; Bilirubin (↑)* Immune response: WBC (↑) ^a ; Lymphocytes (↓) ^a , Monocytes (↑) ^a , Neutrophils (↑) ^a , Eosinophils (↑)*, Orosmuroid (↑) ^a , CRP (–) Iron panel: Ferritin (↑) ^a , Transferrin (↓)*, Iron (↑)* Cardiac muscle damage: cTnT (↑) ^a , proBNP (↑) ^a , Mb (↑) ^a Electrolytes: Na ⁺ (↑) ^a ; Mg ²⁺ (↓) ^a , K ⁺ (↑) ^a , Ca ²⁺ (–)	Post-race (temperate temperature): Blood biochemistry: RBC (↓) ^a ; Hct (↓) ^a ; Hb (↓)*; MCV (–); MCH (↑) ^a ; MCHC (↑) ^a , EPO (↑)* Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; GGT (↑) ^a ; Bilirubin (↑) ^a Immune response: WBC (↑) ^a ; Lymphocytes (↓) ^a , Monocytes (↑) ^a , Neutrophils (↑) ^a , Eosinophils (↓) ^a , Orosmuroid (↑) ^a , CRP (↓) ^a Iron panel: Ferritin (↑) ^a , Transferrin (↑) ^a , Iron (↑)* Cardiac muscle damage: cTnT (↑) ^a , proBNP (↑) ^a , Mb (↑) ^a Electrolytes: Na ⁺ (↑)*; Mg ²⁺ (↓) ^a , K ⁺ (↓)*, Ca ²⁺ (↑) ^a	
Sansoni <i>et al.</i> (2017)	Ultra-marathon (65 km)	17 male participants and 12 age matched, moderately active controls	Blood; before and immediately post-race	Post-race: Bone biochemistry: P1NP (↓) ^a ; Ga-OC (↑)*; Glu-OC (↓) ^a Hormonal response: Glucagon (↑) ^a ; GLP-1 (↑) ^a ; Resistin (↑) ^a ; Visfatin (↑) ^a ; Ghrelin (↑)*; GIP (↑)*Leptin (↓) ^a , C-peptide (↓) ^a ; Insulin (↓)*.		

Zakovska <i>et al.</i> (2017)	Ultra-marathon (100 km; freezing conditions)	15 participants (12 male; 3 female)	Blood; before and immediately post-race	Post-race: Blood cells: RBC (↓)*; Hct (↓)*; Hb (↓)*; PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; ALT (↓)* Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; IgA (↑)*; IgM (↑)*; IgG (↑) ^a ; Lymphocytes (↓)*; Eosinophils (↓)*		
Chlibkova <i>et al.</i> (2018)	Multistage ultra-marathon (n=7 consecutive marathon races)	26 participants (20 male; 6 female)	Blood and urine; before and after stages 1, 4 and 7	After stage 1: Blood biochemistry: Hct (↓) ^a Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (–), Urine: K ⁺ (↑) ^a ; Na ⁺ (↓) ^a	After stage 4: Blood biochemistry: Hct (↓) ^{a,e} Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (↑)* ^d , Urine: K ⁺ (↑) ^{a,d} ; Na ⁺ (↓)*	After stage 7: Blood biochemistry: Hct (↓) ^a Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (↓)*, Urine: K ⁺ (↑) ^{a,c} ; Na ⁺ (–)
Da Ponte <i>et al.</i> (2018)	Marathon (up-hill only)	22 male participants	Blood; before and immediately post-race	Post-race: Blood biochemistry: RBC (–); Hb (–); Hct (↓) ^a ; MCV (↓) ^a ; MCH (↓) ^a ; PL (↑) ^a ; TP (↑) ^a ; Alb (↑) ^a ; MCHC (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a Cardiac muscle damage: cTnI (↑) ^a ; Mb (↑) ^a Electrolytes: K ⁺ (↑) ^a ; Na ⁺ (↓)*; Cl [–] (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; CRP (↑) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ; Basophil (–)		
Li <i>et al.</i> (2018)	Ultra-marathon (400 km)	16 male participants	Blood; before and immediately after the race	Post-race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a		
Liu <i>et al.</i> (2018)	Ultra-marathon (±153.64 km; 24 h)	19 male participants	Blood; before and immediately after	Post-race: Blood biochemistry: Reticulocyte (↑) ^a ; RBC (↓) ^a ; Hct (↓) ^a ; Hb (↓) ^a ; Haptoglobin (↓) ^a ; MCV (↓)*; MCH (↓) ^a ; MCHC (–) Iron panel: Ferritin (↑) ^a ; Iron (↑)*		
Park <i>et al.</i> (2018)	Ultra-marathon (100 km)	22 male participants (11 with EIH and 11 with normal exercise blood pressure)	Blood; before, immediately after, 24 h ⁺ , 72 h ⁺ and 120 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: CRP (↑) ^a		
Shin <i>et al.</i> (2018)	Ultra-marathon (622 km)	10 middle-age male participants	Blood; before, at 300 km, immediately after, as well as 3 ⁺ and 6-days ⁺ post-race	300 km of running: Blood biochemistry: RBC (↓) ^a , PL (↓)*, Hct (↓)*; Hb (↓) ^a Cardiac muscle damage: cTnI (↑) ^a , NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: Hs-CRP (↑) ^a , WBC (↑) ^a	Post-race: Blood biochemistry: RBC (↓) ^a , PL (↑)*, Hct (↓)*; Hb (↓) ^a Cardiac muscle damage: cTnI (↑) ^a , NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: Hs-CRP (↑) ^a , WBC (↑) ^a	

Tirabassi <i>et al.</i> (2018)	Ultra-marathon (100 km)	36 participants (28 male; 8 female)	Blood; before and within 30 min after the race	Post-race: Blood biochemistry: TP (↓) ^a , Alb (↓) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑) ^a Immune response: ALP (↓)*		
Aengevaeren <i>et al.</i> (2019)	Marathon	82 participants (65 male; 17 female)	Blood; before and immediately after the race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; sST2 (↑) ^a		
Bekos <i>et al.</i> (2019)	Marathon	34 marathon participants (24 male; 10 female); 36 half-marathon participants (19 male; 17 female); 30 sedentary volunteers ⁺ (17 male; 13 female)	Blood; before, immediately after as well as 2–7 days ⁺ after the marathon	Half-marathon athletes: Immune response: HSP70 (↑) ^a ; HSP27 (↑) ^a , WBC (↑); PL (↑) – <i>In individuals showing signs of EIB</i>		Marathon athletes: Immune response: HSP70 (↑) ^a ; HSP27 (↑) ^a ; WBC (↑); PL (↑) – <i>In individuals showing signs of EIB</i>
Górecka <i>et al.</i> (2019)	Ultra-marathon	10 male participants	Blood; before, immediately after as well as 90 min post-race ⁺	Post-race: Blood biochemistry: Angiopoietin-like protein 4 (↑) ^a		
Hoppel <i>et al.</i> (2019)	Ultra-marathon (67 km)	8 male participants	Blood; before, after as well as 24 h ⁺ post-race	Post-race: Blood biochemistry: RBC (↑)*, Hct (↑)*; PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Renal damage: BUN (↑) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; CRP (↑) ^a ; Lymphocytes (↓)*; Eosinophils (↓)* Electrolyte markers: Na ⁺ (↓)*; K ⁺ (↑)*; Cl ⁻ (↓)*; Mg ²⁺ (↑)*; Ca ²⁺ (↑) ^a ;		
Le Goff <i>et al.</i> (2019)	Marathon, half-marathon and 1 h duration exercise	23 male marathon participants, 15 male half-marathon participants and male 17 untrained runners ⁺	Blood; before and immediately after each exercise as well as 3 h ⁺ post-race	1 h running: Blood cells: Hb (–) Muscle and hepatic damage: CK (↑) Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑)*; Galactin-3 (↑) ^a ; CK-MB (↑)*; Mb (↑)* Immune response: CRP (↑)*; Myeloperoxidase (↑)* Renal damage: Cystatin (↑)*	Half-marathon: Blood cells: Hb (↑)* Muscle and hepatic damage: CK (↑) Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑) ^a ; Galactin-3 (↑) ^a ; CK-MB (↑)*; Mb (↑)* Immune response: CRP (↑)*; Myeloperoxidase (↑)* Renal damage: Cystatin (↑)*	Post-race: Blood cells: Hb (↑)* Muscle and hepatic damage: CK (↑) Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑) ^a ; Galactin-3 (↑) ^a ; CK-MB (↑)*; Mb (↑)* Immune response: CRP (↑)*; Myeloperoxidase (↑)* Renal damage: Cystatin (↑)*

Martin <i>et al.</i> (2019)	Marathon	22 participants (9 male; 13 female)	Blood; 24 h before, immediately after as well as 24 h ⁺ post-race ⁺	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; BNP (↑) ^a		
Rubio-Arias <i>et al.</i> (2019)	Ultra-marathons (54 km and 11 km)	16 male athletes (54 km n=10; 111 km n=6)	Blood; before and immediately after, as well as 24 h ⁺ , 48 h ⁺ and 72 h ⁺ post-race	After 54 km ultra-marathon: Blood biochemistry: RBC (↑)*; Hb (↑)*; Hct (↑)*; MCV (↑)*; MCH (–); PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a , ALT (↑) ^a , Mb (↑) ^a Cardiac muscle damage: cTnI (↑) ^a Immune response: WBC (↑) ^a , CRP (↓)*	After 111 km ultra-marathon: Blood biochemistry: RBC (↑)*; Hb (↓)*; Hct (↓)*; MCV (↓)*; MCH (↓)*, PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST/GOT (↑) ^a , ALT/GPT (↑) ^a , Mb (↑) ^a Cardiac muscle damage: cTnI (↑)* Immune response: WBC (↑) ^a , CRP (↓) ^a	
Seal <i>et al.</i> (2019)	Ultra-marathon (246 km)	63 male participants	Blood: before, at 93 km and within 15 min after	93 km of running: Electrolytes: Na ⁺ (↓) ^a		Post-race: Electrolytes: Na ⁺ (↓) ^a
Sierra <i>et al.</i> (2019)	Marathon	31 male participants	Sputum (induced); before, immediately after as well as 24 h ⁺ and 72 h ⁺ post-race	Airway immune response; post-race: Neutrophils (↑) ^a ; Macrophages (↑) ^a ; Epithelial cells (↑) ^a ; Lymphocytes (↓)*; IL-6 (↓)*; IL-8 (↓)*; IL-23 (↓) ^a ; IL-33 (↓) ^a ; IL-12p40 (↓) ^a ; TSLP (↓) ^a		
Zebrowska <i>et al.</i> (2019)	Ultra-marathon (24 h; 149 km ± 33 km)	14 male participants	Blood; before, after marathon length as well as 12 h, and 48 h post-race ⁺	Marathon length: Cardiac muscle damage: NT-proBNP (↑) ^a ; CK-MB (↑)*; cTnT (↑)*; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑)*	12 h of running: Cardiovascular damage: NT-proBNP (↑)*, ^c ; CK-MB (↑)*, ^b ; cTnT (↑)*, ^b ; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑) ^{a,d}	Post-race: Cardiovascular damage: NT-proBNP (↑)*, ^c ; CK-MB (↑) ^{a,d} ; cTnT (↑) ^{a,d} ; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑) ^{a,d}
Malek <i>et al.</i> (2020)	Ultra-marathon (100 km)	18 participants (15 male; 3 female)	Blood; before and immediately post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a Immune response: Hs-CRP (↑) ^a		

Footnote: Biochemical changes are denoted as elevated (↑) or reduced (↓), with significance of these changes indicated as follows: significant (^a) and non-significant (*) compared to baseline; non-significant/unreported elevations compared to preceding time point (^b); non-significant/unreported reduction compared to preceding time point (^c); significantly elevated compared to preceding time point (^d), significantly reduced compared to preceding time point (^e), unchanged concentration (–), data not included in the table (+). **Abbreviations:** ACTH: adrenocorticotrophic hormone; AGE: advanced glycation end-products; Alb: albumin; ALP: alkaline phosphatase; ALT: alanine transferase; AST: aspartate transferase; BNP: brain natriuretic peptide; BUN: blood-urea-nitrogen; Ca²⁺: calcium; CK: creatine kinase; CK-MB: creatine kinase-myocardial band; Cl[–]: chloride; CO₂: carbon dioxide; COMP: cartilage oligomeric matrix protein; CRP: C-reactive protein; cTnI: cardiac troponin I; cTnT: cardiac troponin T; CXT: cross-linked C-telopeptide of type I collagen; Dkk-1: dickkopf-1; EIH: exercise-induced hypertension; EPO: erythropoietin; esRAGE: endogenous secretory RAGE; Ga-OC: carboxylate osteocalcin; GGT: gamma-glutamyl transferase; GIP: gastric inhibitory polypeptide; GLP-1: glucagon-like peptide; Glu-OC: undercarboxylated osteocalcin; GM-CFS: granulocyte-macrophage colony-stimulating factor; Hb: haemoglobin; Hct:

haematocrit; H-FABP: heart-type fatty acid-binding protein; HMGB1: high mobility group box 1; Hs-CRP: heat sensitive CRP; HSP: heat shock protein; ICTP: cross-linked carboxy-terminal telopeptide of type I collagen; IFN- γ : interferon-gamma; Ig: immunoglobulin; IL: interleukin; IMA: ischemia-modified albumin; K⁺: potassium; KIM-1: kidney injury molecule-1; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; Mb: myoglobin; MCH: mean corpuscular haemoglobin volume; MCHC: mean corpus haemoglobin concentration; MCV: mean corpus volume; Mg²⁺: magnesium; Na⁺: sodium; NGAL: neutrophil gelatinase-associated lipocalin; NT-proBNP: N-terminal pro-BNP; O₂: oxygen; P1NP: pro-collagen type I N-terminal pro-peptide; P³⁻: phosphate; PICP: procollagen I carboxyterminal pro-peptide; PL: platelets; Pro-ANP: pro-atrial natriuretic peptide; RAGE: receptor of advanced glycation end-products; RANKL: receptor activator of nuclear factor κ B ligand; RBC: red blood cell; sST2: suppression of tumorigenicity 2; TNF- α : tumour necrosis factor alpha; TP: total protein; TSLP: thymic stromal lymphopoietin; VEGF: vascular endothelial growth factor; WBC: white blood cells/leukocytes.

Alternatively, a decrease in Hct has also been associated with haemodilution (Oliveira *et al.*, 2017) due to plasma volume expansion (Schumacher *et al.*, 2002; Robach *et al.*, 2014; Chlibkova *et al.*, 2018; Dos Santos, 2018) and/or overhydration (Knechtle *et al.*, 2009; Hew-Butler *et al.*, 2017) during and after prolonged endurance exercise. Knechtle *et al.* (2008) observed an increase in the total body water percentage in 10 Caucasian athletes after a 17 stage (1 200 km) ER event. Plasma volume expansion is likely subjective to (1) increased protein catabolism (Section 2.3.3), since reduced skeletal muscle mass was also annotated in these athletes; (2) possible renal function impairment (Section 2.2.3); and (3) ER-associated water and renal ion-regulating hormone (aldosterone and vasopressin) adaptations (Knechtle *et al.*, 2008; Chlibkova *et al.*, 2018). Elevated hormone production during and after ER events are regularly reported (Niessner *et al.*, 2003; Siegel *et al.*, 2007; Burge *et al.*, 2011) and are ascribed to the proposed initial system dehydration via excessive sweating or respiration (water and sodium [Na⁺] losses). The resulting reduction in plasma volume and increased plasma osmolality (Knechtle *et al.*, 2008; Burge *et al.*, 2011; Chlibkova *et al.*, 2018), may additionally result in the activation of the renin-angiotensin-aldosterone mechanism (Widmaier *et al.*, 2016; Hew-Butler *et al.*, 2017), and finally the promotion of fluid and Na⁺ re-absorption (plasma volume expansion). This mechanism serves as a preventive measure for a drop in blood pressure and fluid-electrolytes and/or hyponatremia (Hew-Butler *et al.*, 2017), as previously observed (Goudie *et al.*, 2006; Burge *et al.*, 2011; Kipps *et al.*, 2011; Knechtle *et al.*, 2011; Seal *et al.*, 2019). In accordance with the prevention of hyponatremia, Costa *et al.* (2013) investigated the hydration status and Na⁺ adaptations in 74 athletes after completing a five-stage ultra-marathon of 225 km. Despite the fact that the athletes ingested less Na⁺ (± 534 mg/L) than the suggested benchmark parameters (700–1 200 mg/L), the majority were still classified as normonatremic after the ER event, while only eight individuals (11%) presented with asymptomatic exercise-induced hyponatremia (Costa *et al.*, 2013). Seal *et al.* (2019) however, observed mild to severe hyponatremia in 41 of the 65 (63%) sampled athletes immediately after a 245 km ultra-marathon. It is worthy to note, however, that only 14 cases (34%) were associated with severe hyponatremia, while the remaining participants (n=27) developed only mild hyponatremia. As such, it could be suggested that hyponatremia may be dependent on more factors other than fluid intake and hydration status (Knechtle *et al.*, 2011), and factors such as the duration and intensity of the ER exercise bout.

Furthermore, Da Ponte *et al.* (2018), observed significantly elevated blood potassium (K⁺) and reduced chloride (Cl⁻) levels. Although these electrolyte changes (Table 2-1) have been ascribed to dehydration via excessive sweating (Mach & Fuster-Botella, 2017), elevated K⁺ has also been associated with rhabdomyolysis (Da Ponte *et al.*, 2018). Interestingly, elevated K⁺ after ER has been positively correlated with elevated aldosterone (Burge *et al.*, 2011), suggesting a possible association with the aforementioned renin-angiotensin-aldosterone mechanism's role in plasma volume expansion. Since these ions, along with calcium (Ca²⁺), are key components in processes associated with muscle contraction, such as the propagation of action potentials and excitation-contraction coupling (MacLaren & Morton, 2012; Hostrup & Bangsbo, 2017), perturbed homeostasis may result in impaired muscle contraction efficiency, force generation and an overall reduction

in athletic performance. Factors determining the severity of the latter have been linked to age, as well as the intensity, duration and frequency of exercise (Hostrup & Bangsbo, 2017). Although all of these factors could contribute to the discrepancies in the blood parameter adaptations discussed in this section, it is hypothesised that the elapsed time between the cessation of ER and blood sampling could also lead to this variation since longer times allow for better hydration before post-exercise sampling (Kratz *et al.*, 2006).

2.2.2. Cardiovascular markers

Although moderate-intensity exercise is eminent for its beneficial effects on cardiovascular functionality and cardiorespiratory fitness, the degree of exercise intensity that accompanies ER appears to have deleterious effects on these systems (Zebrowska *et al.*, 2019). Cardiovascular infarction or dysfunction and related pathologies are diagnosed using a wide variety of analytical techniques, of which biochemical fluctuations of cardiac troponin T (cTnT) and I (cTnI) are considered the gold standard (Scharhag *et al.*, 2008; Park *et al.*, 2017). Additional biomarkers used for verifying cardiac complications or damage include elevated levels of myoglobin (Mb) and creatine kinase-myocardial band (CK-MB) (Scharhag *et al.*, 2008; Kim *et al.*, 2015), and are generally observed in ER athletes (Table 2-1). However, since CK-MB is not specific to cardiac distress, and has also been associated with skeletal muscle damage (Donnellan & Phelan, 2018), elevations in this marker alone cannot attest to cardiovascular dysfunction in an ER cohort. Park *et al.* (2018) investigated the changes in various cardiac and muscle damage biomarkers in 11 athletes with exercise-induced hypertension (EIH). The results not only supported the elevations in CK-MB and cTnI, but also indicated elevated N-terminal pro-brain natriuretic peptide (NT-proBNP) concentrations. Natriuretic peptides are secreted by the heart in response to volume and wall stretching (Daniels & Maisel, 2007), and have been implicated to be a marker for cardiovascular dysfunction (Di Castelnuovo *et al.*, 2019). Nevertheless, Park *et al.* (2018) ascribed the aforementioned elevated NT-proBNP in athletes with EIH to increased myocardial workload and increased volume pressure associated with EIH, rather than actual cardiovascular dysfunction. Le Goff *et al.* (2019) also indicated elevated brain natriuretic peptide (BNP), in conjunction with increased galectin-3, when comparing the cardiovascular biomarker dynamics of untrained, half-marathon and marathon male athletes immediately post-ER. Similar to BNP, galectin-3 proteins are secreted in response to myocardiocyte stretching, necrosis and cardiac fibrosis (Cheng *et al.*, 2013; Salvagno *et al.*, 2014; Le Goff *et al.*, 2019). Interestingly, Martin *et al.* (2019) observed significantly higher BNP concentrations in female athletes compared to their male counterparts. This suggests increased susceptibility of female athletes to cardiac stress, however, since no correlation could be made between gender and cTnI (Martin *et al.*, 2019), further research is required to establish if females may possess a predisposition pertaining to exercise-induced cardiac stress. Similarly, Leers *et al.* (2006) also detected increased cTnT, BNP and NT-proBNP in athletes (n=27) participating in the Visé-Maastricht-Visé marathon. These increases were however transient, reflecting only temporary myocardial stunning, rather than prolonged damage, and were positively correlated with age, therefore suggesting that these markers may be simultaneously dependent on more than one factor.

Additionally, Zebrowska *et al.* (2019) investigated the acute effects of a 24 h ultra-marathon on novel cardiac markers, e.g. heart-type fatty acid-binding protein (H-FABP) and ischemia-modified albumin (IMA), in order to determine the functionality of these as possible early detection cardiovascular dysfunction biomarkers. Severe elevations in NT-BNP and H-FABP during the 24 h ultra-marathon (particularly at marathon length) supported the phenomenon of myocardial overload and hypoxia-induced myocardial damage and/or increased sarcolemma permeability (Zebrowska *et al.*, 2019), correlating with previous findings (Table 2-1). However, no significant (statistical) difference was observed in the IMA concentrations when compared to baseline values, which was attributed to myocardial protection and the possible increase in antioxidant capacity of athletes who exercise or compete on a regular basis (Zebrowska *et al.*, 2019). Aengevaeren *et al.* (2019) further support the structural toll of ER on cardiac muscle by reporting a significant suppression of tumorigenicity 2, particularly in faster athletes, immediately after a marathon. Although suppression of tumorigenicity 2 is predictive of ventricular overload and myocardial fibrosis, this biomarker is also associated with inflammation (Aimo *et al.*, 2019) and may in this case also indicate elevated inflammatory responses due to exercise-induced rhabdomyolysis.

2.2.3. Muscle, renal and hepatic damage markers

Considering that ER involves a combination of concentric and eccentric muscle contractions, it has been suggested ER inflicts more muscle damage in comparison with other endurance exercises, such as cycling and cross-country skiing (De Oliveira Assumpção *et al.*, 2013). Mechanically, repeated high-forced eccentric contractions in ER primarily disrupt muscle ultra-structure, leading to a cascade of events associated with Ca^{2+} efflux, degradation of structural proteins, and membrane shearing, subsequently releasing numerous muscle-damage associated proteins into the circulatory system (Owens *et al.*, 2019). The aforementioned ultimately leads to cellular apoptosis and/or necrosis, which results in the activation of inflammatory signalling mechanisms to remove cellular debris and initiate repair (Baird *et al.*, 2012; Owens *et al.*, 2019). Lippi *et al.* (2008) investigated the acute biochemical adaptations in 15 Caucasian male athletes after completing a half-marathon and observed significantly elevated creatine kinase and Mb in the post-race samples comparatively, which was attributed to increased membrane permeability due to muscular damage induced by the race. Robach *et al.* (2014) also support the notion of muscle damage as a result of ER and additionally showed elevated concentrations of orosomucoid in 22 male athletes after a 166 km ultra-marathon. This marker plays a role in the acute phase immune response and is known as the anti-fatigue protein that enhances muscle endurance during strenuous activity (Robach *et al.*, 2014; Sun *et al.*, 2018). Animal studies have shown, that orosomucoid is suppressed by oestrogen via p38 mitogen-activated protein kinase in female rats (Sun *et al.*, 2018), thus suggesting that sex may be a determinant factor of athlete muscle performance. Shin *et al.* (2016) investigated biochemical marker fluctuations during three different endurance races (i.e. marathon, 100 km and 308 km) in 15 demographically similar athletes. Elevated lactate dehydrogenase, aldolase, and blood-urea-nitrogen (BUN) were observed and ascribed to muscle and subsequently renal damage (rhabdomyolysis)

(Shin *et al.*, 2016; Hoppel *et al.*, 2019). Both rhabdomyolysis and protein catabolism results in nitrogen release, and consequently the production of urea in the liver (Garrett & Grisham, 2013). Since urea build-up is detrimental to human health, this waste product is removed via the renal system. As such, elevated BUN may be indicative of hepatic and/or renal dysfunction. In accordance with the latter, McCullough *et al.* (2011) observed elevated levels of cystatin C, neutrophil gelatinase-associated lipocalin, kidney injury molecule-1 and serum creatinine (discussed in Section 2.3.3) in 25 athletes who participated in the Flagstar Marathon. These markers are also strongly associated with acute renal injury (Murty *et al.*, 2013; Bonventre, 2014; Khawaja *et al.*, 2019), and elevations in any of these may depict transient renal injury or failure after ER, depending on the exercise severity, which concurs with numerous other investigations (Table 2-1). Furthermore, Rubio-Arias *et al.* (2019), similarly investigated the biochemical adaptations in 16 athletes running 54 km (n=10) or 111 km (n=6), and observed substantially elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations, especially after running 111 km. Although these markers may be indicative of muscle damage, the ratio of ALT to AST (elevated ALT) supports the phenomenon of liver damage, considering ALT is only found in small quantities in muscle cells. These transferases enzymes may also be elevated as both are key components in the metabolic attempt to replenish glucose stores via gluconeogenesis (Section 2.3.3). Considering that these elevations progressively increase concurrently with the distance covered, it may suggest that distance is a depending factor of both muscle and liver damage. This observation was supported by the study of Jastrzebski *et al.* (2015) in which ALT and AST were elevated in 14 athletes after running 25 km, 50 km, 75 km and 100 km. However, these fluctuations were only statistically significant in older athletes at distances >50 km and could be ascribed to the possibility of older subjects possessing reduced hepatic adaptive abilities compared to younger athletes (Jastrzebski *et al.*, 2015). Contradictory to this, however, Tirabassi *et al.* (2018) did not observe any correlation between the elevations of ALT/AST detected and the age, sex, or the rate of distance covered by the athletes (n=36) after a 161 km ultra-marathon. In addition, Shin *et al.* (2016) investigated the adaptations of other hepatic damage-associated markers such as alkaline phosphatase, gamma-glutamyl transferase and bilirubin in athletes after a completing a marathon (n=17), 100 km ultra-marathon (n=17) and 308 km ultra-marathon (n=16). Alkaline phosphatase was significantly elevated following the marathon, with bilirubin only significantly elevated after 100 km, while gamma-glutamyl transferase increased at marathon length and decreased thereafter. This profile may be associated with mild or transient hepatic damage that may correlate with distance. Although the notion of mild or transient hepatic damage is further supported by numerous studies (Table 2-1), this topic seems quite controversial as a select few contrarily reported pronounced and/or prolonged damage (Carvalho *et al.*, 2016; Khan *et al.*, 2018). Nevertheless, these changes unanimously correlate to distance or rate of distance covered, intensity and possibly age.

2.2.4. Immune response and inflammation

The human immune system comprises of two major defence mechanisms namely the; (1) innate and (2) adaptive immune response. The innate immune response focuses on generic protection mechanisms which include localised inflammation, phagocytosis and major histone complex stimulation. The adaptive immune response is mainly enforced by lymphocytes (T-cells, B-cells and immunoglobulins [Ig]) and is more antigen-specific, which is usually activated when the threat or cellular insult has not been neutralised by the innate system (Goh *et al.*, 2019). Physical activity has a profound effect on the immune system (Nieman & Pence, 2019), however, the magnitude of this effect is generally thought to be modulated by the intensity and duration of exercise (Liu *et al.*, 2017a; Sellami *et al.*, 2018). Although moderate-intensity physical activity has been shown to improve the systemic immunity (Nieman, 2011), the intense physical stress of ER is associated with cellular damage, which leads to a cascade of inflammatory responses (Davison *et al.*, 2016; Scheffer *et al.*, 2019). Inflammation is a key defence mechanism regulated by a complex network of pro- and anti-inflammatory cytokines or chemokines to prevent the prevalence of pathogenic diseases and/or internal damage induced by other harmful stimuli such as ER. These cytokines and chemokines may be naturally produced or ingested in the form of non-steroidal anti-inflammatory drugs, which prevents excessive tissue destruction and/or injury (Opal & DePalo, 2000). Excessive inflammation provokes the onset of numerous chronic diseases, including insulin resistance, cardiovascular disease and Alzheimer's disease (Opal & DePalo, 2000; Liu *et al.*, 2017b), only to name a few. Hence, a symbiotic relationship between antagonistic mechanisms (pro- and anti-inflammatory molecules) is required in order to maintain overall health (Barros *et al.*, 2017). In the event of intense endurance activity, inflammation is mainly ascribed to mechanical shearing, reactive oxygen species (ROS)-related damage and various metabolic adaptations. Various biochemical methods have been used to characterise the immunological adaptations induced by prolonged physical activity (as summarised in Table 2-1). Some of the most popular markers associated with inflammation include elevations of C-reactive protein (CRP) and innate immune response white blood cells (WBC; also known as leukocytes) including monocytes, eosinophils, neutrophils, basophils and macrophages (Table 2-1). CRP is produced and stored by hepatocytes and secreted in response to the elevated levels of pro-inflammatory cytokines. Despite structural dissimilarities, this marker possesses similar functions to Ig, particular pertaining to phagocytosis, agglutination and component pathway stimulation (Shrivastava *et al.*, 2015) and is therefore used as an indication of the immune response against a plethora of diseases, such as atherosclerosis and recurrent ischemia (Shrivastava *et al.*, 2015). To this end, Zakovska *et al.* (2017) observed non-significantly (IgA and IgM) and significantly (IgG) elevated Ig in 15 athletes after completing a 100 km ultra-marathon under freezing conditions. On the contrary, numerous studies (Table 2-1) indicated a reduction or suppression of these immune cells and CRP production after ER or exercise. Immunosuppression trends have been associated with a phenomenon known as the 'open window effect' (OWE) (Bekos *et al.*, 2016). The OWE is based on the perception that immune function may be impaired for an extended period after intensive exercise bouts (Goh *et al.*, 2019), increasing the athlete's susceptibility to diseases, particularly to upper respiratory

tract infections (illustrated by the J-curve). As such, the reduction in circulatory immune cells can be ascribed to the relocation of these cells to peripheral tissue (Nieman, 2000; Goh *et al.*, 2019). However, hypotheses such as the J-curve and the OWE have been challenged by various studies (Robson-Ansley *et al.*, 2012; Mach & Fuster-Botella, 2017), possibly debunking the relationship of acute bouts of exercise and immunosuppression (Campbell & Turner, 2018), and giving rise to an alternative model known as the ‘tissue damage or injury model’ (TIM). The TIM proposes that immunosuppression and the subsequent susceptibility to illnesses arise from imbalanced pro- and anti-inflammatory regulation due to an increased training load and frequency, accompanied by insufficient system recovery (Goh *et al.*, 2019). This model also places emphasis on the production of alarmins (damage-associated molecular patterns) in response to exercise-induced tissue or cellular insults, such as apoptosis and necrosis, subsequently stimulating cytokine and chemokine distribution (Yang *et al.*, 2017; Goh *et al.*, 2019). This was supported by Bekos *et al.* (2016) who investigated the biochemical adaptations of the receptor of advanced glycation end-products (RAGE) and suppression of tumorigenicity factor 2, in athletes following a half-marathon (n=36) or marathon (n=34), in comparison to sedentary volunteers (n=30). Serum and urine measurements were collected at baseline, the finish line as well as during recovery, and indicated elevated concentrations of high mobility group box 1 alarmins, advanced glycation end-products (AGE) carboxymethyl-lysine, and soluble RAGE (ligands of AGE), accompanied by altered cytokeratin 18 (CK18) concentrations. Although serum CK18 was elevated in both marathon and half-marathon runners, urinary CK18 was exclusively elevated in the half-marathon participants, whereas caspase-cleaved cytokeratin (ccCK18) remained unchanged in all three cohorts and sample media. Since ccCK18 is only produced as a result of apoptosis of hepatic and renal cells (Li *et al.*, 2017), the isolated elevation in CK18 (parent molecule), may indicate the predominant prevalence of cellular necrosis induced by inflammation rather than apoptosis (Bekos *et al.*, 2016). In addition to the leucocyte and lymphocyte production and stimulation, cellular necrosis releases various molecules (such as the aforementioned alarmins) into circulation, which is crucial for cytokine and chemokine stimulation (Goh *et al.*, 2019), creating a positive feedback regulation loop and the subsequent increase in cellular destruction.

Nielsen *et al.* (2016) investigated cytokine profiles of 40 individuals participating in endurance activities (marathon, half-marathon and a ranger-training course) and observed a substantial increase in interleukin (IL)-6, -8 and -10, as a result of increased WBC counts following muscle shearing, and possibly also the activation of catecholamines (dopamine, epinephrine and norepinephrine), as described by Arakawa *et al.* (2016). However, numerous cytokines, such as tumour necrosis factor- α (TNF- α), interferon-gamma (IFN- γ), vascular endothelial growth factor, IL-2, -4, -5, -12, and -1 β , remained almost unchanged in both ER cohorts, which is ascribed to the complex regulation of pro- and anti-inflammatory cytokines (Nielsen *et al.*, 2016). It was further suggested, that the significant accumulation of the multi-functional IL-6 (pro-inflammatory cytokine and anti-inflammatory myokine) and anti-inflammatory IL-10, might have intercepted the production of TNF- α and IL-1 β (Nielsen *et al.*, 2016), leading to its reduction. On the contrary, however, numerous studies (Table 2-1) have reported significant alterations in the aforementioned cytokines. Sierra *et al.* (2019)

investigated the impairment of cardiopulmonary functionality in 31 male athletes after completing a marathon and observed a negative correlation between cardiopulmonary function and acute inflammatory markers. Significant reductions in the sputum (which was induced) IL-12p40, IL-23, IL-33 and thymic stromal lymphopoietin, accompanied by elevated neutrophils, macrophages, epithelial cells and nitric oxide (NO) exhalation was observed immediately after the marathon. Although these results indicate increased airway inflammation, the reduction of the aforementioned cytokines diminishes the differentiation of T-helper cells (Th) 1, Th2, and Th17, suggesting an imbalanced airway immune response, as well as the possible impediment of cardiopulmonary abilities (Sierra *et al.*, 2019). In light of pulmonary function and inflammation induced by ER, Bekos *et al.* (2019) investigated the prevalence of exercise-induced bronchoconstriction and the role of heat shock proteins (HSPs) in non-asthmatic half-marathon and marathon recreational athletes. HSPs are multi-functional proteins that assist with protein folding, translocation and synthesis, and also act as key regulatory factors secreted in response to stressors such as temperature fluctuations, increased ROS production, inflammation, etc. (Santoro, 2000; Garrett & Grisham, 2013). Significantly elevated concentrations of HSP70 and HSP27 were observed immediately after the ER, however, cohort comparisons indicated larger accumulations of these HSPs in marathon athletes. As such, HSPs, in particular HSP70, is possibly up-regulated due to increased ROS, inflammation and environmental stress induced by ER. However, since these correlate with the fluctuation of WBC concentrations in the athletes, it may also be indicative of a predisposition to exercise-induced bronchoconstriction.

Considering the above, it is clear that inflammation and the overall immune response to ER are not only dependent on, or influenced by, the complex nature of immune regulation, but also the duration and nature of exercise (Elkington *et al.*, 2015; Nieman *et al.*, 2019). When considering the literature (Table 2-1), one could also speculate that cohort fitness, age and sex influence inflammatory marker fluctuations.

2.2.5. Structural damage and associated hormones

Kim *et al.* (2007) investigated the mechanical implications of ER by measuring the alterations in cartilage and muscle damage-associated markers (amongst others) in 54 trained athletes, after a 200 km run. A three-fold increase in cartilage oligomeric matrix protein (COMP) indicated notable cartilage damage, especially during the second half (>100 km) of the race. This was accompanied by elevated creatine kinase (19-fold at 100 km and 90-fold at 200 km) and numerous cytokine markers, which were evident of substantial microfibrillar (a muscle constituent) damage, substantiating the findings discussed in Section 2.2.3. Cartilage damage associated with ER was confirmed by Mündermann *et al.* (2017), who observed elevated COMP levels, even at shorter distances (i.e. marathon length and training distances), in conjunction with elevated heat sensitive-CRP concentrations post-race, both of which correlated with athlete finishing times. Contradictory, Vuolteenaho *et al.* (2014) observed unchanged COMP concentrations in 46 male athletes after completing a marathon, suggesting its dependence on factors other than distance and speed, such as athlete sex, training or fitness status, and the rational of athlete participation (competitive or non-competitive). Furthermore,

Vuolteenaho *et al.* (2014) also investigated ER-induced fluctuations in other cartilage damage-associated markers, such as metalloproteinase-3 (MMP-3), chitinase 3-like protein (YKL-40), and inflammatory-associated adipokines (specifically adiponectin, leptin and resistin). Elevations in MMP-3 and YKL-40 depicted the severe effects of ER on articular cartilage since these markers are regularly observed in patients diagnosed with osteoarthritis (Sharif *et al.*, 2014). The fluctuations in MMP-3 directly correlated with the finishing times of athletes (where faster athletes had higher MMP-3 levels), however, no such correlation could be made with COMP and YKL-40. Significantly elevated adiponectin and resistin, accompanied by the non-significant reduction in leptin, were mainly ascribed to increased inflammatory activity in response to cartilage damage. However, only resistin could be positively correlated with cartilage damage-associated markers, suggesting that resistin may actually be a part of cartilage catabolism during ER. Additionally, considering that resistin is produced by numerous cell types, including osteoblasts (bone formation) and osteoclasts (bone resorption), elevations thereof may also be indicative of adaptations in athlete bone metabolism (Thommesen *et al.*, 2006). Extensive mechanical loading consisting of repeated structure straining and temporary relief thereof (as in the case of ER) has been reported to inflict micro-fatigue damages to bone structures (Kersch-Schindl *et al.*, 2009), collagen type 1 destruction (Sansoni *et al.*, 2017), and adaptations in bone metabolism (Mouzopoulos *et al.*, 2007). This is supported by Kersch-Schindl *et al.* (2015) in a study aimed at determining the effects of a 246 km ultra-marathon on numerous musculoskeletal markers including dkkopf-1, sclerostin, cathepsin, pro-collagen type I N-terminal pro-peptide (P1NP), cross-linked-C-telopeptide of type I collagen (CTX), myostatin and follistatin. The elevated concentrations of both myostatin (inhibition of muscle growth) and its antagonist, follistatin (activation of muscle growth), immediately after the race may be accredited to the dysregulation of muscle metabolism associated extreme overreaching (as in the case of ER). However, the authors also annotated that these elevations could be indicative of rhabdomyolysis (Section 2.2.3) and the fluctuation in haem concentrations associated with ER (Section 2.2.1). Additionally, a significant reduction in P1NP (bone formation marker), accompanied by elevated CTX (bone resorption marker), were observed immediately post-race and ascribed to temporary uncoupled bone metabolism (Kersch-Schindl *et al.*, 2015). Although not observed in this study, it is worth noting, that these bone turnover markers seem to be affected by or dependent on, factors such as age, pregnancy, sex, medication and previous skeletal fractures (Szulc *et al.*, 2017). In addition, reductions in sclerostin (non-significant) and dkkopf-1 could signify an initial activation of osteoblast anabolism since both markers are inhibitors of the Wnt-pathway, responsible for osteoblast commitment/differentiation. However, this profile has also been attributed to possible targeted remodelling and repair of micro-damage (Kersch-Schindl *et al.*, 2015). Furthermore, Kersch-Schindl *et al.* (2009) also observed elevated concentrations of receptor activator of nuclear factor κ B ligand (RANKL) and osteoprotegerin, accompanied by reductions in osteocalcin, within 15 min after completing a 246 km ultra-marathon. Osteoprotegerin and RANKL are natural opponents, considering that RANKL is an activator of osteoclast cells, while osteoprotegerin acts as a decoy receptor for RANKL, thus inhibiting osteoclast production, and in effect bone resorption (Tobeiha *et al.*, 2020). Moreover, a reduction in the hormone osteocalcin indicated an overall reduction in bone turnover (Mouzopoulos *et al.*,

2007; Shin *et al.*, 2012; Sansoni *et al.*, 2017). Although ER acutely reduces bone formation and/or turnover — classifying ER as a negative influence on bone metabolism — this topic remains controversial amongst scientists as some studies have suggested that ER is beneficial in terms of bone turnover and cartilage health in the long-run, as in the case of Lee (2019). In this study, the effect of continuous ER on bone strength and biochemistry was investigated by comparing 68 athletes (recently enrolled in a marathon club) to 40 sedentary individuals. Although no significant difference was observed in deoxypyridinoline (pyridium-links found in collagen type I), a substantial increase in osteocalcin suggests a positive association between endurance activity and bone formation. These findings were intensity and duration dependent, however, athlete sex could also be a contributing factor since female athletes have a higher susceptibility to low mineral bone densities as a part of triad syndrome developed from with chronic ER (Daily & Stumbo, 2018).

Nevertheless, the acute effects of ER on bone metabolism, have also been linked to the perturbed homeostasis of other hormones besides osteocalcin. Mouzopoulos *et al.* (2007) evaluated bone turnover markers, cortisol and parathyroid hormone in 16 athletes after the completion of a 245 km ultra-marathon. Elevated cortisol and parathyroid hormone correlated with reduced osteocalcin concentrations, immediately after the race. Since cortisol and parathyroid hormone reportedly reduce or suppress osteoblast proliferation and function, reduced osteocalcin may result from the elevation of these hormones. Additionally, Sansoni *et al.* (2017) observed fluctuations in adipokines (visfatin, resistin and leptin) and various other hormones (insulin, glucagon, ghrelin, C-peptide, glucagon-like peptide 1 and gastric inhibitory polypeptide) in 17 male athletes, after a mountain ultra-marathon (65 km). Although adipokines are well-known signalling molecules, secreted as a result of various immune responses, some possess the ability to function as metabolic hormones associated with the regulation of fuel substrate catabolism, appetite stimulation, apoptosis, emotional well-being, as well as cellular proliferation and growth (Kurotani *et al.*, 2017). Considering that many of these hormones are related to immune response activation, fluctuations may arise as a result of bone or collagen destruction. However, besides the fact that running extensive distances induces various fluctuations in fuel substrate metabolisms (Section 2.3), bone remodelling is considered to be an energy taxing process which may also contribute to aforementioned hormone changes observed. Evidence of the intertwined nature of this is portrayed by the association between resistin and fat oxidation, visfatin and the regulation of glucose metabolism, nicotinamide adenine dinucleotide (NAD⁺) production and odd-chain fatty acids (OCFA) (Kurotani *et al.*, 2017), as well as leptin and energy homeostasis (Garrett & Grisham, 2013; Hackney, 2019). As such, the aforementioned fluctuations in adipokines and metabolic hormones were associated with increased glucose utilisation (discussed in Section 2.3.1), deregulated insulin mechanisms, and inflammatory response (Sansoni *et al.*, 2017).

2.3. METABOLOMICS AND METABOLOMIC BIOMARKERS

The human metabolome collectively refers to all small intermediates (>1.5 kDa; metabolites) that participate in the metabolic reactions needed for cell function, growth and maintenance (Riekeberg & Powers, 2017;

Nieman *et al.*, 2019). Since the metabolome is a reflection of the genome, transcriptome and proteome, intervention of a stimulus at any of these levels will result in characteristic metabolic changes (Stipetic *et al.*, 2016), subsequently providing a representation of the organism's overall physiological status (Turkoglu *et al.*, 2016). The science of identification and quantification of these metabolites, using highly selective and sensitive analytical instruments, in conjunction with biostatistical analyses, is commonly referred to as metabolomics (De Villiers & Loots, 2013; Heaney *et al.*, 2017). These high-throughput systems typically include but are not limited to, liquid chromatography (LC), capillary electrophoresis, gas chromatography (GC) and nuclear magnetic resonance, which are usually coupled with mass spectrometry (MS), such as quadrupole MS, ion trap MS and time-of-flight MS (TOFMS). The combination of which systems are to be used is determined by the advantages and disadvantages of each, as well as sample complexity, characteristics of the molecules being analysed, aims of the study and the availability of machinery (De Villiers & Loots, 2013). Biostatistical analyses are usually employed to extract significant information, i.e. metabolite markers, from the large datasets obtained and may include a variety of either multi- and/or univariate approaches. Considering the extensive energy-requirements necessary for completing ER events, as well as the physiological and pathological impact that these races have on the human body, metabolomics is a formidable tool when attempting to better characterise the effects of ER.

Energy (in the form of adenosine triphosphate [ATP]) expenditure during physical activity can be described in a hierarchical manner, consisting of three main phases that are mainly dependent on the duration and intensity of the physical activity (MacLaren & Morton, 2012). The first or primary phase consists of phosphocreatine catabolism (intramuscular ATP stores) and provides sufficient energy for approximately 1–10 s at high intensities (MacLaren & Morton, 2012). During the second phase, anaerobic glycolysis is activated to produce lactate and subsequently ATP (Salway, 2012), for approximately 10–60 s of the high-intensity exercise (MacLaren & Morton, 2012). The first two phases provide limited amounts of ATP, while phase three provides the energy required for high-intensity exercise exceeding 60 s via aerobic fuel substrate (carbohydrate, lipid and amino acid [AA]) catabolism (MacLaren & Morton, 2012). As such, most of the studies investigating the effects of endurance exercise on the metabolome of athletes would indicate alterations within the aforementioned fuel substrate catabolic pathways. However, a large portion of the available literature to date is focused on the metabolic adaptations induced by various endurance activities, including rowing (Yan *et al.*, 2009), weightlifting (Sheedy *et al.*, 2014), cycling and ergonomic cycling (Chorell *et al.*, 2012; Nieman *et al.*, 2014; Peake *et al.*, 2014), repeated sprint bouts (Stathis *et al.*, 2006; Pechlivanis *et al.*, 2010; Pechlivanis *et al.*, 2013), voluntary leg extension exercises (Costa *et al.*, 2015), as well as team sports, including rugby (Dubois *et al.*, 2017), leaving a certain void for such studies focused on ER specifically. To this end, Table 2-2 provides a summary of the studies focused on the altered human metabolism induced by ER, with the exclusion of those that are based on animal models and/or the effects of supplements. In the remainder of this literature review, the metabolic changes induced by ER will be discussed in context of the major metabolic fuels and associated pathways affected during ER and the factors that might influence it.

Table 2-2: ER-induced metabolic biomarker adaptations reported in recent (2000–2020) literature, arranged according to the publication year.

Reference	Exercise type	Cohort	Sampling media	Metabolic adaptations	
Mastaloudis, Leonard & Traber (2001)	Ultra-marathon (50 km)	11 participants (8 male; 3 female)	Blood; fasting state, before, at 27 km, immediately after, 1 h ⁺ and 24 h ⁺ post-race	After 27 km of running: Lipid, ketones and cholesterol: Chol (–); TAG (↑)* ROS and vitamins: Urate (↑) ^a ; α-Tocopherol (↑) ^a ; Ascorbate (↑) ^a	Ultra-marathon length Lipid, ketones and cholesterol: Chol (–); TAG (↑)*, ^b ; ROS and vitamins: Urate (↑) ^a ; α-Tocopherol (↑) ^a ; Ascorbate (↑) ^{a,b}
Kratz <i>et al.</i> (2002)	Marathon	37 participants (32 male; 5 female)	Blood; before, within 4 h after and 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Lipid, ketones and cholesterol: TAG (↓)*; Chol (↑)* ROS and vitamins: Urate (↑) ^a ; Cr (↑)	
Petibois <i>et al.</i> (2002)	10 km run	14 participants (<i>sex not provided</i>)	Blood; before and immediately post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a ; Lactate (↑) ^a Lipid, ketones and cholesterol: TAG (↓) ^a ; Glycerol (↑) ^a ; Urea (↑) ^a	
Uchakin <i>et al.</i> (2003)	Marathon (White Rock and Cowton marathons)	15 male participants	Blood; before, 20 min and 60 min after the race as well as 2 ⁺ , 5 ⁺ and 8-days ⁺ post-race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	
Niessner <i>et al.</i> (2003)	Marathon	19 participants (17 male; 2 female)	Blood; before and immediately after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	
Real <i>et al.</i> (2005)	Marathon	22 male participants	Blood; before and after the marathon	Post-race: Carbohydrates and TCA cycle: Glucose (–) Lipid, ketones and cholesterol: FA (↑) ^a ; TAG (↓) ^a Amino acids and urea cycle: Homocysteine (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a , ROS and vitamins: Folate (↑)*; Vit B12 (↓)*	
Leers <i>et al.</i> (2006)	Marathon	27 participants (25 male; 2 female)	Blood; before, immediately after and 24 h ⁺ post-race	Post-race: Amino acids and urea cycle: Urea (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a	
Kim <i>et al.</i> (2007)	Ultra-marathon (200 km)	54 male participants	Blood; before, after 100 km and immediately post-race	Post-race: Carbohydrates and TCA cycle: Lactate (↑)*; Glucose (↓)* Purine, pyrimidine and ATP: Cr (↑) ^a	

Mouzopoulos <i>et al.</i> (2007)	Ultra-marathon (245 km)	16 male participants	Blood; 5-days before, immediately after as well as 1 ⁺ , 3 ⁺ and 5-days ⁺ post-race	Post-race: Amino acids and urea cycle: OH-proline (↓) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	
Siegel <i>et al.</i> (2007)	Marathon	33 participants (<i>sex not provided</i>)	Blood; before and after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a	
Weibel & Glonek (2007)	Ultra-marathon (>65 km per day for 6 days)	31 participants	Urine; before and 4-20 times during the race	Post-race (average): Lipid, ketones and cholesterol: Ketones (↑) ^a (n=22); Ketones (↓) ^a (n=9)	
Lehmann <i>et al.</i> (2010)	Treadmill exercises (±14 km -16 km)	21 participants	Blood; before and immediately after exercise	Post-race: Carbohydrates and TCA cycle: Lactate (↑) ^a Lipid, ketones and cholesterol: Hippurate (↑) ^a ; C6:0 car. (↑) ^a ; C8:0 car. (↑) ^a ; C10:1 car. (↑) ^a ; C10:0 car. (↑) ^a ; C14:2 car. (↑) ^a ; C12:0 car. (↑) ^a	
Lewis <i>et al.</i> (2010)	Marathon and 60 min treadmill running exercises	25 marathon participants and 45 individuals (treadmill exercise)	Blood; before and after exercise	Treadmill exercise: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Malate (↑) ^a ; Succinate (↑) ^a ; Fumarate (↑) ^a ; Pyruvate (↑) ^a ; Glucose-6-phosphate (↑) ^a ; 3-Phosphoglycerate (↑) ^a Lipid, ketones and cholesterol: Glycerol (↑) ^a Amino acids and urea cycle: Alanine(↑) ^a ; Leucine (↑) ^a ; Serine (↓)*; Glutamate (↓) ^a ; Cysteine (↓) ^a ; Homocysteine (↓) ^a ; Glutamine (↓) ^a ; Citrulline (↓) ^a Purine, pyrimidine and ATP: Inosine (↑) ^a ; Hypoxanthine (↑) ^a ; AMP (↑) ^a ; Xanthine (↑) ^a ; Allantoin (↓) ^a ; Niacin (↑) ^a ; Pantothenate (↑) ^a ; Cr (↑)*	Marathon distance: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Malate (↑)*; Aconitate (↑) ^a ; α-ketoglutarate (↑) ^a ; Citrate (↑) ^a ; Succinate (↑) ^a ; Fumarate (↑) ^a ; Pyruvate (↑) ^a ; Glucose-6-phosphate (↑) ^a Lipid, ketones and cholesterol: Glycerol-3-phosphate (↑) ^a ; Glycerol (↑) ^a ; β-OH-butyrate (↑) ^a Amino acids and urea cycle: Leucine (↓) ^a ; Serine (↓) ^a ; Glutamine (↓) ^a ; Proline (↓) ^a ; Lysine (↓) ^a ; Threonine (↓) ^a ; Betaine (↓) ^a ; Asparagine (↓) ^a ; Dimethylglycine (↓) ^a ; Histidine (↓) ^a ; Kynurenate (↑) ^a ; Valine (↓) ^a ; Homovanillate (↑) ^a ; 4-OH-phenylpyruvate (↑) ^a ; Arginine (↓) ^a ; Quinolate (↑) ^a ; Tryptophan (↓) ^a ; Citrulline (↓) ^a ; Ornithine (↓) ^a ; Argininosuccinate (↑) ^a Purine, pyrimidine and ATP: Hypoxanthine (↑) ^a ; AMP (↑) ^a ; IMP (↑) ^a ; Xanthine (↑) ^a ; Xanosine (↑) ^a ; Niacin (↑) ^a ; Pantothenate (↑) ^a ; Cr (↑)*
McCullough <i>et al.</i> (2011)	Marathon	25 participants (12 male; 13 female)	Blood: before, after and 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a	

Turner <i>et al.</i> (2011)	Ultra-marathon (233 km)	9 male participants	Blood; before, immediately after as well as 24 h ⁺ , 7 ⁺ and 28-days ⁺ post-race	Post-race: ROS and vitamins: Glutathione (↑) ^a ; Lipid peroxides (↑)		
Waśkiewicz <i>et al.</i> (2012)	Ultra-marathon (168.5 km)	19 male participants	Blood; before, at marathon distance, within 12 h and immediately after the ultra-marathon	Post-race: Lipid, ketones and cholesterol: Chol (–); TAG (–); FA (↑) ^a ; Glycerol (↑) ^a ; β-OH-butyrate (↑)*	12 h of running: Lipid, ketones and cholesterol: Chol (–); TAG (–); FA (↑) ^{a,b} ; Glycerol (↑) ^{a,c} ; β-OH-butyrate (↑) ^{a,c}	Post-race: Lipid, ketones and cholesterol: Chol (↓) ^a ; TAG (↓) ^a ; FA (↑) ^{a,c} ; Glycerol (↑) ^{a,c} ; β-OH-butyrate (↑) ^{a,b}
Klapcinska <i>et al.</i> (2013)	Ultra-marathon (48 h; 183–320 km)	7 male participants	Blood; before, after running 12 h, 24 h and within 10 min after the race as well as 24 h ⁺ and 48 h ⁺ post-race	12 h of running: Carbohydrates and TCA cycle: Lactate (↓)*; Glucose (↓)*	24 h of running: Carbohydrates and TCA cycle: Lactate (↓)* ^c ; Glucose (↑)*	Post-race: Carbohydrates and TCA cycle: Lactate (↓)* ^c ; Glucose (↑)* ^b
Nieman <i>et al.</i> (2013)	Repeated treadmill exercises (±25 km per day for 3 days)	15 participants (7 male; 8 female)	Blood; before and directly after the 3-day intensified training exercise	Post-exercise: Carbohydrates and TCA cycle: Succinate (↑) ^a ; Fumarate (↑) ^a ; Malate (↑) ^a Lipid, ketones and cholesterol: Palmitoleate (↑) ^a ; Myristoleate (↑) ^a ; 10-heptadecenoate (↑) ^a ; 10-nonadecenoate (↑) ^a ; Dihomolinoleate (↑) ^a ; Stearidonate (↑) ^a ; Myristate (↑) ^a ; Eicosenoate (↑) ^a ; Oleate (↑) ^a ; Margarate (↑) ^a ; Laurylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; 4-decenoylcar. (↑) ^a ; Dodecanedioate (↑) ^a ; Hexadecanedioate (↑) ^a ; Tetradecanedioate (↑) ^a ; Octadecanedioate (↑) ^a ; Linolenate (↑) ^a ; Docosapentaenoate (↑) ^a ; Linoleate (↑) ^a ; Eicosapentaenoate (↑) ^a ; Dihomolinolenate (↑) ^a ; Docosahexaenoate (↑) ^a Lysolipid, bile salts and steroids/hormones: 1-arachidonoylGPEA (↓) ^a ; 1-palmitoylGPEA (↓) ^a ; 1-oleoylGPEA (↓) ^a ; 2-linoleoylGPEA (↓) ^a ; Taurodeoxycholate (↓) ^a ; Taurocholate (↓) ^a ; Cholate (↓) ^a ; Glycochenodeoxycholate (↓) ^a ; Glycodeoxycholate (↓) ^a ; Glycocholate (↓) ^a ; 2-aminoadipate (↑) ^a Amino acids and urea cycle: 4-OH-phenyl-pyruvate (↑) ^a ; N-acetylphenylalanine (↑) ^a ; Isovalerylcar. (↑) ^a ; OH-isobutyrate (↑) ^a		
Khodaei <i>et al.</i> (2015)	Ultra-marathon (161 km)	20 participants pre-marathon (14 male; 6 female), 21 participants post-marathon (17 male; 4 female)	Blood; before and immediately after	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a		

Daskalaki <i>et al.</i> (2015)	1 h treadmill/bicycle ergometer exercise	3 male participants	Urine; before (first pass) ⁺ , 1 h before ⁺ , immediately after as well as numerous times within the preceding 24 h ⁺ post-exercise	Post-exercise: Carbohydrates and TCA cycle: Pyruvate (↑)^a Purine, pyrimidine and ATP: N2-N2-Dimethylguanosine (↑)*; Xanthosine (↑)^a; Inosine (↑)^a; Deoxyinosine (↑)^a; Guanine (↑)^a; Hypoxanthine (↑)^a Amino acids and urea cycle: 3-OH-tryptophan (↑)*; Xanthurenate isomer (↑)^a; Kynurenate (↑)*; OH-tryptophol (↓)*; N1-Methyl-2-pyridone-5-carboxamide (↑)*; Tryptophan (↓)*; O-Acetyl-L-homoserine (↑)^a; Histidine (↓)*; Methionine (↑)*; Proline (↑)*; Acetylvanilalanine (↑)^a; N-(Carboxyethyl) arginine (↑)^a; N-Acetyl-D-tryptophan (↑)^a; N-acetylmethionine (↑)*; Indole pyruvate (↑)*; Acetamidooxohexanoate (↑)*; OH-phenylpyruvate (↑)*; Oxoarginine (↑)*; Guanidovaleramide (↑)^a; AcetoOH-butanoate (↑)^a; Acetamidobutanoate (↑)*; DiOH-methylbutanoate (↑)*; Methyl-oxopentanoate (↑)*; Dioxopentanoate (↑)*; OH-pentanoate (↑)*; Methyloxobutanoate (↑)*; OH-butanoate(↑)* ROS and vitamins: Riboflavin (↑)*; Pantothenate (↑)^a Lipid, ketones and cholesterol: Dodecenoylcar. (↑)^a; Undecanoylcar. (↑)*; Decanoylcar. (↑)*; L-Octanoylcar. (↑)*; Methylglutarylcar. (↑)*; Hexanoylcar. (↑)*; Valerylcar. (↑)*; DeOH-car. (↑)^a Lysolipid, bile salts and steroids/hormones: Urocortisol glc. (↑)*; Dihydrocortisone glc. (↑)*; Hydrocortisone sulphate (↑)*; OH-androsterone glc. (↑)*; Androstane diol glc. (↑)*; Androsterone glc. (↑)*; Oxoandrostane glc. (↑)*	
Suzuki <i>et al.</i> (2015)	5 h running exercise (Participant A= 34.14 km; participant B=43.05 km)	2 female participants	Blood; before, within 60 min after as well as 1 ⁺ , 3 and 5 h post-race	Participant A post-race: Carbohydrates and TCA cycle: Glucose (↑) Lysolipid, bile salts and steroids/hormones: Cortisol (↓); Adrenalin (↑); Noradrenalin (↑); Dopamine (↑) Lipid, ketones and cholesterol: TAG (↓), Acetoacetate (↑), 3-OH-butyrate (↑) Amino acids and urea cycle: Aspartate (↓); Glutamate (↑); Asparagine (↑); Serine (↑); Glutamine (↑); Glycine (↑); Histidine (↑); Arginine (↑); Taurine (↓); Threonine (↓); Alanine (↑); Proline (↑); Tyrosine (↑); Valine (↑); Methionine (↑); Isoleucine (↑); Leucine (↑); Phenylalanine (↑); Lysine (↑)	Participant B post-race: Carbohydrates and TCA cycle: Glucose (↑) Lysolipid, bile salts and steroids/hormones: Cortisol (↑); Adrenalin (↑); Noradrenalin (↑); Dopamine (↑) Lipid, ketones and cholesterol: TAG (↓), Acetoacetate (↑), 3-OH-butyrate (↑) Amino acids and urea cycle: Aspartate (↓); Glutamate (↓); Asparagine (↓); Serine (↓); Glutamine (↓); Glycine (↓); Histidine (↓); Arginine (↓); Taurine (↓); Threonine (↓); Alanine (↓); Proline (↓); Tyrosine (↑); Valine (↓); Methionine (↑); Isoleucine (↓); Leucine (↑); Phenylalanine (↓); Lysine (↓)
Mrakic-Sposta <i>et al.</i> (2015)	Ultra-marathon (330 km)	25 male participants	Blood and urine; before, during (±165 km) and immediately post-race	After 165 km of running: Carbohydrates and TCA cycle: Lactate (↑)^a; Glucose (↑)* Lipid, ketones and cholesterol: Ketones (–) Purine, pyrimidine and ATP: Cr (–) ROS and vitamins: Neopterin (–); ROS production rate (↑)^b; 8-OH-2-deoxyguanosine (–); 8-isoprostane (–)	Post-race: Carbohydrates and TCA cycle: Lactate (↑)^a; Glucose (↑)* Lipid, ketones and cholesterol: Ketones (↑)^a Purine, pyrimidine and ATP: Cr (↑)^a ROS and vitamins: Neopterin (↑)^a; ROS production rate (↑)^a; 8-OH-2-deoxyguanosine (↑)^a; 8-isoprostane (↑)^a

Arakawa <i>et al.</i> (2016)	Ultra-marathon (130 km)	18 middle-aged participants	Blood; before, after 42 km (marathon), immediately after as well as 3 ⁺ , 5 ⁺ and 7-days ⁺ post-race	<u>Marathon length:</u> Lipid, ketones and cholesterol: FA (↑); TAG (↓)*; Chol (↑)*; HDL-C (↑)* Lysolipid, bile salts and steroids/hormones: Cortisol (↑)*; Adrenalin (↑) ^a ; Noradrenalin (↑) ^a ; Dopamine (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)		<u>Post-race:</u> Lipid, ketones and cholesterol: FA (↑) ^a ; TAG (↓) ^a ; Chol (↓); HDL-C (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a ; Adrenalin (↑) ^{a,b} ; Noradrenalin (↑) ^{a,c} ; Dopamine (↑)* ^c Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*
Bekos <i>et al.</i> (2016)	Marathon and half-marathon	34 marathon (24 male; 10 female), 36 half-marathon participants (19 male; 17 female) and 30 sedentary controls (17 male; 13 female)	Blood and urine; before and immediately after as well as 2–7 days ⁺ post-race	<u>Half-marathon:</u> Carbohydrates and TCA cycle: Lactate (↑) ^a Purine, pyrimidine and ATP: Urine Cr (–); Serum Cr (↑) ^a		<u>Marathon:</u> Carbohydrates and TCA cycle: Lactate (↑) ^a Purine, pyrimidine and ATP: Urine Cr (–); Serum Cr (↑) ^a
Shin <i>et al.</i> (2016)	Ultra-marathon (308 km)	15 participants (<i>sex not provided</i>)	Blood; before, at marathon distance, after 100 km and immediately post-race	<u>Marathon length:</u> Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) ^a	<u>100 km of running:</u> Purine, pyrimidine and ATP: Cr (↑) ^{a,d} ; Urate (↑) ^{a,d}	<u>Post-race:</u> Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*
Traiperm <i>et al.</i> (2016)	Marathon	50 adolescent participants (30 male; 20 female)	Blood; before, immediately after as well as 24 h ⁺ post-race	<u>Post-race:</u> Carbohydrates and TCA cycle: Glucose (↑) ^a Lipid, ketones and cholesterol: TAG (↓) ^a ; HDL (↓) ^a ; LDL (↓) ^a ; Chol (↓) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*		
Vezzoli <i>et al.</i> (2016)	Ultra-marathons (50 km and 100 km)	24 participants (50 km n=6 [3 male; 3 female] and 100 km n=18 [7 male; 11 female])	Blood; before and immediately after the race	<u>After 50 km:</u> ROS and vitamins: Plasma: Cysteine (↑)*; Homocysteine (↑)*; Cysteinylglycine (↑)*; Glutathione (↑)*; TBARS (↑) ^a ; ROS (↑) ^a ; Erythrocyte: Cysteine (↑)*; Homocysteine (↑)*; Cysteinylglycine (↑)*; Glutathione (↑)*; 8-OH-2-deoxyguanosine (↑) ^a ; 8-isoprostane (↑) ^a		<u>After 100 km:</u> ROS and vitamins: Plasma: Cysteine (↑) ^a ; Homocysteine (↑) ^a ; Cysteinylglycine (↑) ^a ; Glutathione (↑) ^a ; TBARS (↑) ^a ; ROS (↑) ^a ; Erythrocyte: Cysteine (↑)*; Homocysteine (↑)*; Cysteinylglycine (↓)*; Glutathione (↑)*; 8-OH-2-deoxyguanosine (↑) ^a ; 8-isoprostane (↑) ^a
Yamada <i>et al.</i> (2016)	Ultra-marathon (135 km)	35 male participants	Blood; before, after 45 km and immediately post-race	<u>45 km of running:</u> Lipid, ketones and cholesterol: FA (↑) ^a Amino acids and urea cycle: Tryptophan (↓)*; Serotonin (↑) ^a ; Kynurenine (↓)*		<u>Post-race:</u> Lipid, ketones and cholesterol: FA (↑) ^{a,d} Amino acids and urea cycle: Tryptophan (↓) ^a ; Serotonin (↓)*, ^e ; Kynurenine (↑)*, ^d

Nieman <i>et al.</i> (2017)	Treadmill exercise (±24.9 km)	24 male participants	Muscle biopsy and blood; before and after exercise	Post-exercise: Carbohydrates and TCA cycle: Glycogen (↓) ^a ; Succinate (↑) ^a ; Malate (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a ; Adrenalin (↑) ^a Lipid, ketones and cholesterol: Glycerol (↑) ^a ; Myristate (↑) ^a ; Oleate (↑) ^a ; Margarate (↑) ^a ; 16-OH-palmitate (↑) ^a ; 10-heptadecenoate (↑) ^a ; Myristoleate (↑) ^a ; Palmitoleate (↑) ^a ; Palmitate (↑) ^a ; Dihomolinoleate (↑) ^a ; Eicosenoate (↑) ^a ; Laurate (↑) ^a ; 10-nonadecenoate (↑) ^a ; Linoleate (↑) ^a ; Linolenate (↑) ^a ; Hexadecanedioate (↑) ^a ; Stearate (↑) ^a ; Docosadienoate (↑) ^a ; Nonadecanoate (↑) ^a ; 5-dodecenoate (↑) ^a ; Stearidonate (↑) ^a ; Arachidate (↑) ^a ; Pentadecanoate (↑) ^a ; 3-OH-butyrate (↑) ^a ; Caprate (↑) ^a ; Decanoylcar. (↑) ^a ; Methionylalanine (↑) ^a ; Tetradecanedioate (↑) ^a ; Octanoylcar. (↑) ^a ; Acetoacetate (↑) ^a ; 3-OH-decanoate (↑) ^a ; Docosapentaenoate (↑) ^a ; 13/9-HODE (↑) ^a ; 17-methylstearate (↑) ^a ; Laurylcar. (↑) ^a ; Decenoylcar. (↑) ^a ; Adrenate (↑) ^a ; Octadecanedioate (↑) ^a ; Erucate (↑) ^a ; 10-undecenoate (↑) ^a ; Methylpalmitate (↑) ^a ; 3-OH-octanoate (↑) ^a ; Dodecanedioate (↑) ^a	
Danese <i>et al.</i> (2017)	Half-marathon	30 participants (21 male; 9 female)	Blood; before and immediately after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cholate (↓) ^a ; Deoxycholate (↓) ^a ; Chenodeoxycholate (↓) ^a ; Ursodeoxycholate (↓) ^a ; Taurochenodeoxycholate (↑)*; Glycocholate (↓)*; Glycochenodeoxycholate (↓)*; Glycoursodeoxycholate (–); Glycodeoxycholate (–); Hyodeoxycholate (↓) ^a ; Lithocholate (–); Taurocholate (–)	
Oliveira <i>et al.</i> (2017)	Marathon (hot and temperate temperatures)	26 male participants	Blood and urine, before, immediately after as well as 1 ⁺ and 3-days ⁺ after	Post-race (Heat): Amino acids and urea cycle: Urea (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) ^a ; TBARS (↑) ^a ; SOD3 (↑) ^a ; Catalase (↓)*	Post-race (Temperate): Amino acids and urea cycle: Urea (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*; TBARS (↑) ^a ; SOD3 (↓)*; Catalase (↓)*
Davison <i>et al.</i> (2018)	1 h treadmill exercise (75% VO _{2max} ; Hypoxic and normoxic conditions)	24 male participants	Blood; before, immediately after as well as 3 h post-exercise ⁺	Post-exercise (Hypoxia): Lipid, ketones and cholesterol: Propionylcar. (↑) ^a ; Butyrylcar. (↑) ^a ; 2-Methylbutyrylcar./Pivaloylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Undecenoylcar. (↑) ^a ; 4,8 dimethylnonanoyl car. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Tridecenolcar. (↑) ^a ; Tetradecadienoylcar. (↑) ^a ; cis-5-Tetradecenoylcar. (↑) ^a ; Hexadecatetraenoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadec-2-enoyl car. (↑) ^a ; Palmitoylcar. (↑) ^a ; Octadecatrienoylcar. (↑) ^a ; Elaidic car./Vaccenyl car. (↑) ^a ; Hippurate (↑) ^a ; Myristate (↑) ^a ; Palmitoleate (↑) ^a ; α-Linolenate (↑) ^a ; Linoleate (↑) ^a ; Arachidonate (↑) ^a	Post-exercise (Normoxia): Lipid, ketones and cholesterol: Propionylcar. (↑) ^a ; Butyrylcar. (↑) ^a ; 2-Methylbutyrylcar./Pivaloylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Undecenoylcar. (↑) ^a ; 4,8 dimethylnonanoyl car. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Tridecenolcar. (↑) ^a ; Tetradecadienoylcar. (↑) ^a ; cis-5-Tetradecenoylcar. (↑) ^a ; Hexadecatetraenoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadec-2-enoyl car. (↑) ^a ; Palmitoylcar. (↑) ^a ; Octadecatrienoylcar. (↑) ^a ; Elaidic car./Vaccenyl car. (↑) ^a ; Hippurate (↑) ^a ; Myristate (↑) ^a ; Palmitoleate (↑) ^a ; α-Linolenate (↑) ^a ; Linoleate (↑) ^a ; Arachidonate (↑) ^a

Da Ponte <i>et al.</i> (2018)	Marathon (up-hill only)	22 male participants	Blood; before and immediately post-race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a
Howe <i>et al.</i> (2018)	80.5 km treadmill exercise (self-paced)	9 male participants	Blood; before and immediately post-exercise	Post-exercise: Carbohydrates and TCA cycle: Rhamnose (↓) ^a ; Galactosamine (↓) ^a ; Hexose (↓) ^a Lipid, ketones and cholesterol: Acetylcar. (↑) ^a ; Propanoylcar. (↑) ^a ; Butanoylcar. (↑) ^a ; Hexenoylcar. (↑) ^a ; Hexanoylcar.isomer (↑) ^a ; Heptanoylcar. (↑) ^a ; Octenoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Nonanoylcar. (↑) ^a ; Decadienoylcar. (↑) ^a ; Decenoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Keto-decanoylcar. (↑) ^a ; Dimethylnonanoylcar. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Hydroxydodecanoylcar. (↑) ^a ; Tetradecadienylcar.isomer (↑) ^a ; Tetradecanoylcar. (↑) ^a ; Hydroxytetradecadienylcar. (↑) ^a ; Hydroxytetradecenoylcar. (↑) ^a ; Hydroxymyristoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadecenoylcar. (↑) ^a ; Palmitoylcar. (↑) ^a ; Hydroxyhexadecadienoylcar. (↑) ^a ; Hydroxyhexadecenoylcar. (↑) ^a ; Octadecadienoylcar. (↑) ^a ; Hexadecanedioicacid monocar. ester (↑) ^a ; Decanoate (↑) ^a ; Dodecadienoate (↑) ^a ; Dodecanoate (↑) ^a ; Decanedioate (↑) ^a ; Hydroxydodecatrienoate; Tridecenoate (↑) ^a ; Tetradecadienoate (↑) ^a ; Tetradecenoate iso (↑) ^a ; Tetradecenoate iso (↑) ^a ; Dodecanedioate (↑) ^a ; Hydroxytetradecadienoate (↑) ^a ; Pentadecenoate (↑) ^a ; Hydroxytetradecadienoate (↑) ^a ; Hydroxytetradecanoate iso (↑) ^a ; Hydroxytetradecanoate iso (↑) ^a ; Hexadecadienoate iso (↑) ^a ; Hexadecadienoate iso (↑) ^a ; Palmitoleate (↑) ^a ; Tetradecanedioate (↑) ^a ; Hydroxyhexadecatrienoate (↑) ^a ; Hydroxyhexadecadienoate (↑) ^a ; Heptadecenoate (↑) ^a ; Hydroxyhexadecenoate iso (↑) ^a ; Hydroxyhexadecenoate (↑) ^a ; Hydroxyhexadecanoate (↑) ^a ; Octadecatetraenoate (↑) ^a ; Linolenate (↑) ^a ; Linoleate (↑) ^a ; Oleate (↑) ^a ; Dihydroxyhexadecadienoate (↑) ^a ; Dihydroxyhexdecenoate (↑) ^a ; Hydroxyoctadecadienoate (↑) ^a ; Hydroxyoctdecenoate (↑) ^a ; Dihydroxyoctadecanoate (↑) ^a ; Nitrooctadecenoate (↑) ^a ; Docosahexaenoate (↑) ^a ; Docosapentaenoate (↑) ^a ; Docosatetraenoate (↑) ^a Amino acids and urea cycle: Glycine (↓) ^a ; Alanine (↓) ^a ; 3-Amino-isobutanoate (↓) ^a ; Serine (↓) ^a ; Pyrrole-2-carboxylate (↓) ^a ; Proline (↓) ^a ; Oxopentanoic acid (↓) ^a ; Guanidinoacetate (↓) ^a ; Valine (↓) ^a ; Betaine (↓) ^a ; Hydroxypentanoate (↑) ^a ; Threonine (↓) ^a ; Taurine (↓) ^a ; 5-Oxoproline (↓) ^a ; Hydroxyproline (↓) ^a ; Leucine (↓) ^a ; Isoleucine (↓) ^a ; Hydroxyhexanoic acid (↑) ^a ; Asparagine (↓) ^a ; Ornithine (↓) ^a ; Urocanate (↓) ^a ; Glutamine (↓) ^a ; Lysine (↓) ^a ; Glutamate (↓) ^a ; Methionine (↓) ^a ; Imidazol-5-yl-pyruvate (↓) ^a ; Indole-3-acetaldehyde (↓) ^a ; O-Acetylhomoserine (↓) ^a ; Arginine (↓) ^a ; Citrulline (↓) ^a ; Tyrosine (↓) ^a ; Hydroxyphenyllactate (↓) ^a ; Acetyl-L-lysine (↓) ^a ; Kynurenate (↑) ^a ; Tryptophan (↓) ^a ; Formylhydroxykynurenamine (↓) ^a ; Hydroxyindolepyruvate (↑) ^a ; 1-Methylnicotinamide (↓) ^a Lysolipid, bile salts and steroids/hormones: Hydrocortisone (↑) ^a ; Urocortisone (↑) ^a ; Hydroxycholanate (↓) ^a ; Deoxycholanate (↓) ^a ; Chenodeoxyglycocholate (↓) ^a ; Glycocholate (↓) ^a ; Taurocholate (↓) ^a ; Chenodeoxycholate glc. (↓) ^a ; Cholestane—tetrol-glc. (↓) ^a ROS and vitamins: γ-Tocopherol (↓) ^a ; α-Tocopherol (↓) ^a Purine, pyrimidine and ATP: Hypoxanthine (↑) ^a ; Uridine (↓) ^a ; Pseudouridine (↓) ^a

Li <i>et al.</i> (2018)	Ultra-marathon (400 km)	16 male participants	Blood; before and immediately after the race	Post-race: Amino acids and urea cycle: Alanine (↓)^a; Proline (↓)^a; Valine (↓)^a; Leucine (↓)^a; Isoleucine (↓)^a; Ornithine (↓)^a; Citrulline (↓)^a; Arginine (↑)*; Tryptophan (↓)*; Histidine (↑)*; Lysine (↓)^a; Methionine (↑)^a; Phenylalanine (↑)^a; Threonine (↓)^a; Glycine (↓)^a; Serine (↓)*; Taurine (↑)^a; Aspartate (↑)*; Glutamine (↑)*; Glutamate (↑)*; Cystathionine (↑)^a; Cystine (↑)^a; Homocysteine (–); 1-Methyl-L-histidine(↓)*; 3-Methyl-L-histidine (↑)*; α-Aminonbutyrate (↓)*; Argininosuccinate (↑)^a; β-Alanine (↑)*; Carnosine (↑)^a; Ethanolamine (↑)*; Homocitrulline (↓)*; OH-lysine (↓)*; Phosphorylethanolamine (↑)^a; Tyrosine (↑)^a; α-Aminoadipate (↑)*; Asparagine (↓)*; Phosphoserine (↓)^a; Sarcosine (↓)^a Lysolipid, bile salts and steroids/hormones: Testosterone (↓)^a; Progesterone (↓)^a; 17-α-OH-progesterone (↓)^a; 11-Deoxycortisol (↓)*; 11-Deoxycorticosterone (↓)*; Cortisol (↑)^a; Aldosterone (↑)^a; Dehydroepiandrosterone (↓)*; Dehydroepiandrosterone sulphate (↑)^a; 4-Androstenedione (↓)^a; Dihydrotestosterone (↓)*; Corticosterone (↑)*; Cortisone (↓)*; Estrone (↑)^a; Oestradiol (↑)*
Stander <i>et al.</i> (2018)	Marathon	31 participants (19 male; 12 female)	Blood; before and immediately post- race	Post-race: Carbohydrates and TCA cycle: Arabitol (↑)^a; Citrate (↑)^a; Erythritol (↑)^a; Fumarate (↑)^a; Glucarate (↑)^a; Glucose (↑)^a; Malate (↑)^a; Mannitol (↑)^a; Mannose (↑)^a; Myo-inositol (↑)^a; Pyruvate (↑)^a; Sorbose (↑)^a; Succinate (↑)^a; Tagatose (↑)^a; Threonate (↑)^a Lipid, ketones and cholesterol: α-OH-octanoate (↑)^a; α-Linolenate (↑)^a; β-OH-butyrate (↑)^a; OH-hexanoate (↑)^a; β-OH-α,β-didehydrosebacate (↑)^a; 5-Dodecenoate (↑)^a; 5-Pregnene-3β,20α-diol (↑)^a; 10-Heptadecenoate (↑)^a; 11-Eicosenoate (↑)^a; 11,14-Eicosadienoate (↑)^a; Acetoacetate (↑)^a; Glycerol (↑)^a; Heptadecanoate (↑)^a; Laurate (↑)^a; Linoleate (↑)^a; Malonate (↑)^a; Myristoleate (↑)^a; Oleate (↑)^a; Palmitate (↑)^a; Palmitoleate (↑)^a; Pentadecanoate (↑)^a; Monopalmitin (↑)^a; Squalene (↑)^a; Tridecanoate (↑)^a Amino acids and urea cycle: α-Aminomalonate (↑)^a; α-Ethylhydracrylate (↑)^a; β-OH-isobutyrate (↑)^a; β-OH-isovalerate (↑)^a; β-OH-propionate (↑)^a; Alanine (↓)^a; Aspartate (↓)^a; Dimethylglycine (↓)^a; Glutarate (↓)^a; Glycerate (↑)^a; Glycine (↓)^a; Indole-3-acetate (↓)^a; Leucine (↓)^a; Methionine (↓)^a; Phenylalanine (↓)^a; p-OH-phenylacetate (↑)^a; p-OH-phenyllactate (↑)^a; Pyroglutamate (↓)^a; Serine (↓)^a; Threonine (↓)^a; Tyrosine (↓)^a; Valine (↓)^a
Górecka <i>et al.</i> (2019)	Ultra-marathon	10 male participants	Blood; before, immediately after as well as 90 min post-race ⁺	Post-race: Lipid, ketones and cholesterol: Glycerol (↑)^a; TAG (↓)^a; Chol (↑)*; FA (↑)^a
Malek <i>et al.</i> (2020)	Ultra-marathon (100 km)	18 participants (15 male; 3 female)	Blood; before and immediately post- race	Post-race: Carbohydrates and TCA cycle: Glucose (↑)*; Lactate (↑)*

Schader <i>et al.</i> (2020)	Marathon	76 male participants	Blood; before (one week), immediately after as well as 24 h ⁺ and 72 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Hexoses average (↓)^a Lipid, ketones and cholesterol: C2:0–C18:0 car. (↑)^a; C2:1–C6:1 car. (↑)^a C10:1–C18:1 car. (↑)^a; C10:2 car. (↑)^a; C10:2 car. (↑)^a; C10:2 car. (↑)^a; C10:2 car. (↑)^a; C10:2 car. (↑)^a; C10:2 car. (↑)^a; C14:2 car. (↑)^a; C14:2OH car. (↑)^a; C16:2 car. (↑)^a; C16OH car. (↑)^a; C18:2 car. (↑)^a; C18:1OH car. (↑)^a; C3OH car. (↑)^a; C5OH car. (↑)^a; C6 car. (↑)^a; LysoPC C14:0 (↓)^a; LysoPC C16:0 (↓)^a; LysoPC C16:1 (↓)^a; LysoPC C17:0 (↓)^a; LysoPC C18:0 (↓)^a; LysoPC C18:1 (↓)^a; LysoPC C18:2 (↓)^a; LysoPC C20:3 (↓)^a; LysoPC C20:4 (↓)^a; LysoPC C24:0 (↓)^a; LysoPC C28:0 (↓)^a; LysoPC C28:1 (↓)^a; PC C26–C44 (↓)^a; SMOH C14:1 (↓)^a; SMOH C16: (↓)^a; SMOH C22:1 (↓)^a; SMOH C22:2 (↓)^a; SMOH C24:1 (↓)^a; SMOH C16:0 (↓)^a; SMOH C18:0 (↓)^a; SMOH C24:0 (↓)^a; SM C16:1 (↓)^a SM C18:1 (↓)^a SM C20:2 (↓)^a SM C22:3 (↓)^a SM C24:1 (↓)^a SM C26:1 (↓)^a Amino acids and urea cycle: Alanine (↓)^a; Arginine (↓)^a; Glutamine (↓)^a; Glutamate (↑)^a; Histidine (↓)^a; Leucine (↓)^a; Lysine(↓)^a; Methionine (↓)^a; Ornithine (↑)^a; Citrulline (↓)^a; Phenylalanine (↓)^a; Proline (↓)^a; Serine (↓)^a; Threonate (↓)^a; Tryptophan (↓)^a; Tyrosine (↓)^a; Valine (↓)^a
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Footnote: Significance of adaptations are indicated as follows: significant (^a) and non-significant (*) compared to baseline; non-significantly/unreported elevations compared to preceding time point (^b); non-significantly/unreported reduction compared to preceding time point (^c); significantly elevated compared to preceding time point (^d), significantly reduced compared to preceding time point (^e), unchanged concentration (–), data not included in the table (⁺). **Abbreviations:** AMP: adenosine monophosphate; ATP: adenosine triphosphate; Car: carnitine; Chol: cholesterol; Cr: creatine; FA: fatty acids; Glc: glucuronide; GPEA: glycerophosphoethanolamine; HDL: high-density lipoprotein; IMP: inosine monophosphate; Iso: isomer; LDL: low-density lipoprotein; OH: hydroxy; PC; Phosphatidylcholine; ROS: reactive oxygen species; SOD: superoxide dismutase; SM: Sphingomyelins; TAG: triacylglycerol; TBARS: thiobarbituric acid reactive substances; TCA: Tricarboxylic acid; Vit: vitamin.

2.3.1. Carbohydrate metabolism and the tricarboxylic acid (TCA) cycle

Carbohydrates (muscle glycogen and glucose) are considered to be the primary fuel substrates utilised by the body during physical activity (Stellingwerff, 2012), making it a key component in the regulation of athletic performance. Glucose obtained or released from gluconeogenesis as well as intramuscular (muscle glycogen stores) and extra-muscular (liver glycogen and gastrointestinal absorption) sources are used as fuel substrates during physical activity, where the preference for usage depends on the intensity of exercise (MacLaren & Morton, 2012). As in the case of endurance exercise ($W_{\max} \geq 75\%$), muscle glycogen accounts for most of the glucose catabolised. However, these stores are limited and may become “depleted” (Nieman *et al.*, 2017; Hearnis *et al.*, 2018) after approximately 90 min of exercise at $VO_{2\max} \geq 75\%$ (Stellingwerff, 2012), affecting athletic performance. Lewis *et al.* (2010) employed a targeted (n=42 metabolites) LC-MS metabolomics approach and observed elevated concentrations of various glycolysis-associated intermediates (pyruvate, glucose-6-phosphate, lactate and niacinamide) in 25 athletes after a marathon. Elevated glycolysis intermediates detected immediately after ER indicates the upregulation of glucose catabolism, a finding which is supported by numerous other studies (Table 2-2). Furthermore, accumulated lactate and pyruvate, not only further substantiate the elevated lactate dehydrogenase levels observed (Table 2-1) but also indicate a possible reduction in oxygen availability in muscles cells, since higher lactate concentrations are observed when exercising under hypoxic conditions (Sumi *et al.*, 2019). Excessive lactate production is neutralised by reacting with bicarbonate (from renal excretion) to produce carbonic acid and eventually carbon dioxide (Salway, 2012). This concurs with the reported reductions in circulating bicarbonate levels after ER (Sumi *et al.*, 2019) and may be associated with post-exercise hyperventilation, impaired glomerular filtration rate or renal function (Section 2.2.3), and metabolic acidosis (Kratz *et al.*, 2002; Salway, 2012; Waśkiewicz *et al.*, 2012). However, since hydrogen ions are generated via glycolysis, subsequently leading to lower pH levels, a reduction in the bicarbonate pool may be further ascribed to the strain place on the glycolysis, regardless of lactate production (Salway, 2017; Robergs *et al.*, 2018). Nevertheless, elevated lactate concentrations may also be interpreted as an attempt to regenerate oxidised NAD^+ needed in the glycolysis pathway and TCA cycle (Salway, 2012), when an imbalanced redox potential is apparent (White & Schenk, 2012). The latter could also cause the dramatic changes in the TCA cycle intermediates (malate, fumarate, α -ketoglutarate, aconitate, citrate/iso-citrate and succinate) observed by Lewis *et al.* (2010), Nieman *et al.* (2017) and Stander *et al.* (2018). Since the TCA cycle is the central point of influx for substrate catabolism, increased production of NADH (reduced form of NAD^+) and flavin adenine nucleotide ($FADH_2$), is expected due to the immense strain placed on the electron transport chain (ETC) in an attempt to produce sufficient ATP during such activities (Salway, 2012). This, in turn, may result in NADH/ $FADH_2$ accumulation, NAD^+ /FAD ‘depletion’ and the transient suppression of TCA cycle enzymes (isocitrate dehydrogenase, citrate synthase and α -ketoglutarate dehydrogenase), in turn causing a slight halt in the cycle flow, and eventually the accumulation of TCA cycle precursor intermediates (Garrett & Grisham, 2013; Esterhuizen *et al.*, 2017). However, an imbalanced

NAD⁺/NADH redox potential after ER seems to remain controversial, and depend on numerous factors such as exercise duration, intensity and hydration status (White & Schenk, 2012).

Furthermore, Petibois *et al.* (2002) reported a slight increase in glucose concentrations in 14 athletes after a 10 km endurance run, which concurred with previous literature (Table 2-2), and found a positive correlation between capillary blood glucose concentrations and athletic performance. Stander *et al.* (2018) hypothesised that this may be ascribed to the initial reduction in glycogen and glucose within 90 min after the start of high intensity (>75% VO_{2max}) exercise, possibly resulting in a reduction in insulin secretion and/or sensitivity (Kim *et al.*, 2007; Arakawa *et al.*, 2016). Kirwan (2010) suggested that transient inhibition of insulin-dependent components and/or signalling pathways (i.e. glucose transporter type 4, insulin receptor substrate-1 and various kinases) may occur during extensive eccentric muscle contractions. Since some of these components are vital in terms of glucose absorption into cells, extracellular accumulation of glucose can be expected and explain the fluctuations in gluconeogenic intermediates detected after ER (see Table 2-2). However, since ER usually extend over a prolonged period (particularly during marathons and ultra-marathons), athletes would typically ingest large amounts of carbohydrate-containing foods and beverages, which impact their metabolic profiles, as observed by more robust studies that did not control dietary intake (Jeukendrup, 2011; Pfeiffer *et al.*, 2012; Stander *et al.*, 2018). Waśkiewicz *et al.* (2012) for instance, investigated the acute metabolic adaptations induced by a 24 h (±168.5 km) ultra-marathon race in 14 male athletes. When measuring blood glucose levels after the athletes reached marathon distances, non-significant reductions or unchanged glucose concentrations were reported, supporting the results of various other studies (Table 2-2). However, blood glucose levels started rising at 75 km and were highest after the full ultra-marathon distance, suggesting that these fluctuations may be distance-dependent. Suzuki *et al.* (2015) additionally attributed fluctuations in glucose to be dependent on an athlete's individual ability to metabolically adapt to endurance exercise.

2.3.2. Lipid metabolism

Another fuel substrate utilised during high-intensity prolonged exercise, are lipids, that originate from various sources, including adipose tissue, muscle fibres (intra-muscular), cholesterol and dietary triacylglycerols (TAG) (Purdom *et al.*, 2018). Since lipids are considered high-energy content substrates, catabolism thereof is thought to be prominent when glucose and muscle glycogen absorption and catabolism are transiently hampered/insufficient to provide enough energy for the duration of the endurance exercise. Activation of lipid catabolism is mediated by an integrated system of catecholamines, cortisol, growth hormones, glucagon and insulin (inhibition) (Purdom *et al.*, 2018), further substantiating the fluctuations in these hormones as reported on numerous occasions (Table 2-2). Górecka *et al.* (2019) further annotated reduced TAG concentrations, accompanied by elevated glycerol, fatty acids (FA; Table 2-2) and angiopoietin-like protein 4, in 10 male athletes after a 100 km ultra-marathon. This profile is supported by various other investigations (Table 2-2) that strongly indicate increased lipolysis activity with subsequent energy production via β -oxidation and gluconeogenic influx (Salway, 2012). Although lipolysis occurs in the cytoplasm of cells, β -oxidation is

confined to the mitochondrial matrix, which is surrounded by a set of membranes that are almost impenetrable for long-chain FAs (LCFAs) (Garrett & Grisham, 2013). As such, LCFAs, and in some cases medium-chain FA (activated by acyl-CoA synthetase), are transformed to acyl-carnitines by an outer mitochondrial membrane-associated enzyme known as carnitine palmitoyl transferase (CPT) I, whereafter an inner membrane-associated translocase facilitates the acyl-carnitine transport into the mitochondria matrix (Salway, 2012; Garrett & Grisham, 2013). Once on the inside of the matrix, CPT II is responsible for the removal of the carnitine component, and the resulting acyl-CoA (Garrett & Grisham, 2013), is further oxidised by β -oxidation (Salway, 2012). In accordance, Davison *et al.* (2018) used an LC-based serum metabolomics approach to characterise the metabolome changes induced by ER under hypoxic, conditions in 24 male athletes completing a 1h ER session. They observed significantly elevated concentrations of a wide array of carnitines and LCFAs. Although this may merely exploit FA-transport limitations via CPT-I (Purdom *et al.*, 2018), an alternative explanation may include a saturated/impaired β -oxidation pathway due to the high metabolic strain of high-intensity exercise. This is based on the principle that β -oxidation possesses a rate-limiting β -hydroxyacyl dehydrogenase step, consequently giving rise to maximum fat oxidation rates, which have been reported to be optimal at intensities of 45–65% $\text{VO}_{2\text{max}}$ (Purdom *et al.*, 2018). This may be further substantiated by the study of Schader *et al.* (2020), in which higher concentrations of acyl-carnitines and CPT were apparent in less fit/slower athletes (running at lower intensities of 50–60% $\text{VO}_{2\text{max}}$) in comparison to the more fit/faster athletes, thus exploiting the dependence of lipolysis during lower intensity exercise. As such, performing prolonged exercises at intensities exceeding that of the optimal fat oxidation rate could lead to an accumulation of FA or acyl-carnitines, and the subsequent activation of alternative FA acids catabolic pathways. The latter is supported by elevated (peroxisomal and mitochondrial) β -oxidation, ω -oxidation and α -oxidation intermediates observed by numerous other studies (Table 2-2). This is further depicted by elevated concentrations of various OCFA observed by Stander *et al.* (2018) in 16 marathon athletes, using an untargeted GCxGC-TOFMS metabolomics approach. However, OCFA may also originate from cellular membrane autophagy and/or dietary ingestion (Jenkins *et al.*, 2017; Stander *et al.*, 2018).

Up-regulated lipid catabolism directly correlates with increased ketogenesis, since acetyl-CoA is considered the substrate for the latter. Ketones are utilised by the brain and skeletal muscles when glucose/glycogen are significantly reduced (Cahill & Vech, 2003; Meidenbauer *et al.*, 2015), as apparent after 60-90 mins after the start of ER. This supports the elevated ketones and ketone-bodies observed after engaging in ER. However, Weibel and Glonek (2007) demonstrated that ketone body production may be subjective in endurance athletes, as 9 out of 31 athletes in their study did not appear to produce additional ketones after an ultra-marathon. The researchers attributed this to possible fluctuations in pre-race carbohydrate ingestion and/or the possibility of alternative adaptive metabolic mechanisms in some athletes (Volek *et al.*, 2016). Furthermore, they also hypothesised that the biological medium used (i.e. urine) could have influenced the results (Weibel & Glonek, 2007). It is also important to note that some of the ketone bodies in the studies supporting ketone production

after ER (Table 2-2) are not necessarily products of acetyl-CoA transformation, but rather end-products from branched-chain AA (BCAA) metabolism (discussed in Section 2.3.3).

Besides the traditional catabolism of TAG, cholesterol is also considered a viable fuel substrate during ER (Purdom *et al.*, 2018). This lipid-sterol is not only one of the major constituents of cellular membranes, but is also the precursor/substrate for bile salt and steroidogenesis pathways (Salway, 2012). As such, elevations in squalene (Stander *et al.*, 2018), cortisol (Mouzopoulos *et al.*, 2007; Arakawa *et al.*, 2016) and other natural steroid-associated metabolites (Daskalaki *et al.*, 2015; Nieman *et al.*, 2017; Howe *et al.*, 2018; Stander *et al.*, 2018) are regularly observed in literature (Table 2-2). These natural steroid hormones produced from cholesterol (pregnenolone, progesterone, cortisol, etc.) are key components in fuel substrate catabolism mediation as well as energy homeostasis (Salway, 2012; Garrett & Grisham, 2013). Furthermore, Nieman *et al.* (2013) used an untargeted GC-MS and LC-MS metabolomics approach to identify the metabolome adaptations in 15 runners after a three-day training period (2.5 h per day; ± 25.9 km). Reduced concentrations of 22 lysolipid/bile acid metabolites were observed immediately post-exercise, supporting the findings of numerous other studies (Danese *et al.*, 2017; Howe *et al.*, 2018). It was hypothesised that these cholesterol-associated alterations may be due to the aforementioned autophagy of cellular membranes, as well as the preferential production of steroid hormones necessary for fuel substrate absorption or catabolism, thus reducing the availability of cholesterol for alternative pathways such as bile production.

2.3.3. AA metabolism

Once strenuous physical activity continues to the point that glycogen stores are “depleted” and FA oxidation has reached a maximal plateau, the metabolism of free AA and muscle protein degradation. This is supported by significant reductions in various AAs, and elevations in AA-associated catabolism intermediates (Table 2-2). Some of these AA intermediates may also be principal components in pathways not directly related to energy production (Salway, 2012; Garrett & Grisham, 2013). In particular, Yamada *et al.* (2016) reported reduced concentrations of tryptophan, accompanied by elevated kynurenine and serotonin in 35 Japanese athletes after completing a 48 h ultra-marathon. Although these fluctuations mainly correlated with distance and intensity, kynurenine is also suggested to induce central fatigue, while the increased serotonin (mainly after 45 km) is associated with gluconeogenesis, lipolysis and increased phosphofructokinase activity, which is apparent in athletes (Yamada *et al.*, 2016). However, a reduction in serotonin after running 135 km may be ascribed to an increased ROS production, since the latter inhibits tryptophan hydroxylase, lowering tryptophan and elevating kynurenine production (Yamada *et al.*, 2016), as these are opposing catabolism pathways (Salway, 2012). Real *et al.* (2005), detected elevated concentrations of homocysteine (methionine catabolism intermediate), which is associated with increased risk of cardiovascular dysfunction or malfunction, as indicated by various aforementioned physiologically and immunologically inclined studies (Section 2.2.2). Moreover, numerous studies (Table 2-2) observed elevated concentrations of *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetic acid (phenylalanine and tyrosine intermediates) immediately post-marathon. These

metabolites are usually indicative of mild hepatic damage, which correlate with other studies (Section 2.2.3) that reported increased concentrations of hepatic transamination enzymes, including ALT and AST (Table 2-1). Both of these transamination enzymes are imperative in AA transamination oxidation reactions, which produce aspartate, a precursor of the urea cycle. Further substantiation of increased AA transamination during ER, is indicated by Lewis *et al.* (2010) and Petibois *et al.* (2002), as both identified elevated urea cycle metabolites, including urea, argininosuccinate and creatine, accompanied by reduced levels of ornithine and citrulline, immediately following strenuous activity. Reduced ornithine and citrulline may potentially result from arginase inhibition by N^G-hydroxy-L-arginine, an intermediate produced during elevated NO synthesis (Morris, 2002). The increased argininosuccinate and creatine concentrations most likely occur as a result of increased AA transaminase activity and the body's need to replenish creatine stores. Creatine is a storable form of ATP (in the form of the aforementioned phosphocreatine) and may spontaneously be converted to creatinine after the release of the energy from the high energy phosphate bond (Salway, 2012). Therefore, elevated serum creatinine (Table 2-2) has been associated with increased muscle breakdown and, in combination with the aforementioned elevation in BUN levels (Section 2.2.3), are indicative of a reduced glomerular filtration rate due to renal damage (McCullough *et al.*, 2011; Salway, 2012). Traiperm *et al.* (2016) supported this finding in a study aimed at investigating the biochemical effects of marathon running on 50 adolescent athletes (30 males and 20 females). During this investigation, a positive correlation was observed between increased creatinine levels post-marathon and athlete sex, however, no such a correlation could be established with finishing times, indicating that creatinine circulation is comparable across various performance levels (Traiperm *et al.*, 2016).

In addition, the reduced BCAAs (leucine, isoleucine and valine) and associated α -ketoacids observed by numerous studies (Table 2-2), indicating increased BCAA oxidation as an alternative fuel substrate (Li *et al.*, 2018). Leucine, among other factors (oxygen, AMP and growth factors), is a key regulator of the mammalian target of rapamycin complex 1 (mTORC I) signalling pathway, which is a natural inhibitor of autophagy (Laplane & Sabatini, 2009). As such, Stander *et al.* (2018) proposed that reduced leucine concentrations may inhibit mTORC 1, in turn activating autophagy to release additional membrane-embedded fuel substrates. Leucine also regulates insulin release from pancreatic β -cells when glucose levels become low (Salway, 2012; Kujala *et al.*, 2013), which concurs with the aforementioned transient hampering of insulin secretion. From a neurometabolic perspective, BCAAs are vitally important for the regulation of neurotransmitter (NT) synthesis and transport since catabolism of these are considered nitrogen donors required for excitatory NT (glutamate and γ -aminobutyric acid) production in the brain, the blood-brain transportation of various compounds are highly restricted (Garrett & Grisham, 2013), and BCAAs compete with various NT precursor metabolites for diffusion into the brain (IOF, 2011). Several major classes of NTs have been identified, including cholinergic AA and derivatives of these peptides, and catecholamines (Garrett & Grisham, 2013). Of these, Arakawa *et al.* (2016) reported elevated concentrations of the catecholamines including noradrenalin, adrenalin and dopamine in 18 middle-aged athletes completing marathon and ultra-marathon distances. Since catecholamine

production is dependent on various AA precursors, these elevations could also be linked to the aforementioned reductions AA.

2.3.4. Purine metabolism, ROS and vitamins

Since the major energy molecules (ATP and guanosine triphosphate [GTP]) utilised in the metabolism are constructed from purine bases (Garrett & Grisham, 2013), it is anticipated that severe energy-requiring states would cause alterations within the purine catabolism pathway. This is depicted by urinary elevations in deoxyinosine, inosine, guanine, xanthosine, and hypoxanthosine in an untargeted LC-MS metabolomics study aimed at investigating whether a 1 h aerobic endurance exercise session would impose metabolic alterations or not (Daskalaki *et al.*, 2015). Additional studies (Zielinski *et al.*, 2009; Lewis *et al.*, 2010) further annotated elevations in AMP, inosine-monophosphate, hypoxanthine, xanthine, urate, and allantoin within 60 min after exercise started. Besides ATP and GTP (muscle nucleotide) degradation (Davison *et al.*, 2018), these fluctuations have also been associated with reduced internal oxygen (hypoxia) and elevated ROS (Zielinski *et al.*, 2009; Lewis *et al.*, 2010; Garrett & Grisham, 2013; Daskalaki *et al.*, 2015). One of the many proposed mechanisms by which purine degradation may lead to increased ROS production includes the upregulation of xanthine oxidase (Gomez-Cabrera *et al.*, 2007) during muscle contractions (Steinbacher & Eckl, 2015). This enzyme is responsible for the conversion of hypoxanthine to xanthine and the subsequent production of urate (Salway, 2012; Garrett & Grisham, 2013), thus substantiating the elevated concentrations of urate observed (Table 2-2). In the event that ROS production exceeds the body's ability to counteract these using antioxidants, oxidative stress is increased and may lead to deleterious effects (Ozcan & Ogun, 2015). Mrakic-Sposta *et al.* (2015) investigated elevated ROS, oxidative damage, inflammatory markers (Section II.4) and antioxidant capacity in 46 male athletes before, during, and after a mountain ultra-marathon. Significant elevations in mitochondrial ROS were observed in these athletes after the race, which was associated with excessive ETC leakage due to the tremendous strain placed on the mitochondria for energy production (Mrakic-Sposta *et al.*, 2015). This group also observed elevated levels of 8-hydroxy-2-deoxyguanosine (8-OH-2dG), 8-OH-dG and 8-isoprostane, indicating increased DNA nucleotide oxidation (Turner *et al.*, 2011) and lipid peroxidation due to oxidative stress. Similarly, Vezzoli *et al.* (2016) investigated the correlation between oxidative stress and the duration of ER in athletes competing in a 50 km (n=6) and 100 km (n=18) ultra-marathon. The authors further annotated significantly elevated oxidised plasma amino thiols (cysteine and cysteinyl-glycine), oxidised glutathione, and thiobarbituric acid-reactive substances, after the ER events. Considering that elevations in oxidised antioxidants were only deemed statistically significant at 100 km, it may suggest that redox status is dependent on ER duration and intensity. The elevations in thiobarbituric acid-reactive substances, which are reportedly robust markers associated with lipid peroxidation, have been regularly observed (Klapcinska *et al.*, 2013; Oliveira *et al.*, 2017; Howe *et al.*, 2018) following ER and support previous findings of impaired redox status. Furthermore, Mastaloudis *et al.* (2001) observed an increase in vitamin E (tocopherol) clearance in 11 athletes after completion of a 50 km ultra-marathon due to lipid peroxidation, explaining the reductions in

tocopherol-intermediates observed previously (Howe *et al.*, 2018). However, although Traber (2007) indicated a positive correlation between vitamin E supplements and reduced lipid peroxidation, no such correlation could be established in terms of reduced muscle damage, DNA damage, inflammation and other oxidative stress-associated effects. Moreover, vitamin B5 (pantothenate) was also previously detected in significantly elevated concentrations following ER (Lewis *et al.*, 2010; Lustgarten *et al.*, 2013). Vitamin B5 is one of eight B vitamins which assists in energy production by converting carbohydrates into fuel (i.e. glucose) and is also necessary for the proper functionality of the nervous system, and utilisation of fats and proteins for energy.

2.4. IN A NUTSHELL, WHAT DOES THIS MEAN?

2.4.1. The problem and its roots

Despite previous attempts to elucidate the systematic biochemical effects of ER (listed in Table 2-1 and Table 2-2), a definite conclusion whether it is overall more deleterious or beneficial to human health remains unresolved. This may largely be ascribed to the fact that no human-based study can compensate for the various inevitable phenotypic and genotypic factors. In synopsis of the reviewed studies, blood parameters were highly subjective to hydration status (i.e. overhydration and dehydration), whereas cardiovascular, muscle, renal, hepatic, and structural (bone) alterations, and subsequent immune responses, were mostly dependent on the duration and intensity of an acute bout of ER, age, sex, and the fitness status of athletes. Metabolically, dietary regimens or preferences (both during and pre-exercise), along with an athlete's ability to adapt to vigorous metabolic switching, seems to add to these the various controversies among research groups. In terms of athlete sex correlations, is clear from Table 2-1 and Table 2-2 that very few studies included female athletes, which may be attributed to the increased susceptibility of female athletes to hormonal fluctuations, female triad syndrome and subsequently cardiovascular disease. Female triad syndrome is associated with reduced energy availability and bone mineral density, accompanied by menstrual dysfunction (Daily & Stumbo, 2018). Nevertheless, considering that female athlete participation in endurance races increases exponentially per annum, future research should include more female participants in cohorts. Additionally, less frequently described variation factors, such as (1) fitness level (not based on experience), (2) detailed notation of recovery period since the last ER event or training session, (3) the effects of the short resting periods or a reduction in exercise intensity during repeated bouts, and (4) participant psychological rationale (competitive race determinants vs non-competitive treadmill exercise) should also be taken into account.

2.4.2. A possible proficiency classification system

Considering that athletic proficiency and exercise intensity/duration are some of the most influential factors determining biomarker adaptations during and after ER, adequate standards for a uniform classification are of utmost importance. Although Stoggl and Sperlich (2015) provided a clear structure for classifying exercise intensity, to our knowledge, no such standard is available for classifying ER athletes according to investigative

distance-specific proficiency. As such, we propose a structured proficiency classification system (Table 2-3), collectively based on the literature presented in Table 2-1 and Table 2-2. Classification should firstly be based on the investigative distance of the specific experimental protocol. Hereafter, proficiency can be assigned based on two of the following three factors, either individually or when taking the average of the entire cohort into account: (1) total/average “years of experience” in terms of participation or training, (2) the (average) “number of competitive endurance races” per annum (p.a.), and/or (3) “weekly training km” (average). For example, when investigating the effects of a marathon in athletes with four years of experience, who have competed in an average of five endurance races, and whom train by running an average of 35 km weekly, the following classification would be made based on Table 2-3: A marathon would be classified as “level 3”, and based on “experience” and/or “number of competitive endurance races” and/or “weekly training” (any two of these factors), these athletes would be classified as “intermediate athletes”. In the event that two of the three criteria per investigative distance are not applicable to the athletes (either individually or when considering the cohort average), we suggest basing proficiency on the average “weekly training km” of athletes only, thus avoiding bias towards competitive vs non-competitive athletes.

Table 2-3: Proposed proficiency classification system for ER investigations.

Investigative distance level	Novice athletes (0≤2yrs experience)		Intermediate athletes (3<5yrs experience)		Master athletes (5<10yrs experience)		Elite athletes (≥10yrs experience)	
	Number of competitive endurance races p.a.	Weekly training (km)	Number of competitive endurance races p.a.	Weekly training (km)	Number of competitive endurance races p.a.	Weekly training (km)	Number of competitive endurance races p.a.	Weekly training (km)
Level 1 (0<15 km)	0–2	0.0–3.5	3–5	3.5<7.0	5–10	7.0<10.5	10≤	≥10.5
Level 2 (15<30 km)		<18.5		18.5<22.0		22.0<25.5		≥25.5
Level 3 (30<50 km)		<35.0		35.0<40.0		40.0<45.0		≥45.0
Level 4 (50<80 km)		<57.5		57.5<65.0		65.0<72.5		≥72.5
Level 5 (80<150 km)		<87.5		87.5<95.0		95.0<102.5		≥102.5
Level 6 (150<300 km)		<180.7		180.7<225.0		225.0<262.5		≥262.5
Level 7 (300 km≤)		<350.0		350.0<400.0		400.0<450.0		≥450.0

2.5. RECOVERY

Considering the potentially detrimental nature of some of these effects, recovery becomes the next important consideration in assuring athlete health and optimal performance. In this particular instance, the generally accepted definition of “recovery” is the collective restoration of the disrupted functionality of a system, to a pre-exercise related state (Tomlin & Wenger, 2001; de Jonge *et al.*, 2020), and primarily encompasses the repair of molecular and structural damages, replenishment of fuel substrate reserves and the restoration of fluid imbalances (Peake, 2019). Repeated failure to meet individual recovery requirements after exercise, may result in non-functional overreaching, and subsequently overtraining syndrome (Kenttä & Hassmén, 1998; Bandyopadhyay *et al.*, 2012). Besides performance impairment, overtraining syndrome evokes severe fatigue, an elevated resting heart rate, psychological disturbances, as well as reduced immunity and the subsequent increased susceptibility to disease (Kenttä & Hassmén, 1998; Nieman *et al.*, 2018; de Jonge *et al.*, 2020). Whilst the precise time required for sufficient systematic recovery is dependent on various factors such as athlete fitness, dietary regimens, and the duration and time of the running exercise, recovery is usually attained within two days to two weeks after cessation of the exercise, with the exception of athletes with overtraining syndrome, in which this recovery period may be further prolonged to months (Bandyopadhyay *et al.*, 2012).

2.5.1. The basics of unaided metabolic recovery

Considering that the metabolome is the downstream product of the genome, transcriptome and proteome (Wishart, 2019), convalescence of all the effects presented at these respective levels may be concurrent with the recovery of the metabolism (Tomlin & Wenger, 2001). Although the precise mechanism(s) of metabolic recovery after strenuous exercise remains to be elucidated, it is generally thought to proceed in a biphasic manner, facilitated by the excess oxygen consumption and slightly increased metabolic rates that are apparent within the immediate hours post-exercise (Tomlin & Wenger, 2001; Egan & Zierath, 2013). The initial phase encompasses the swift restoration of tissue oxygen, phosphocreatine supplies, as well as the removal of blood lactate, while the succeeding phase further entails the recovery of fuel substrate reserves and innate metabolite adaptations (Tomlin & Wenger, 2001). However, it has been suggested that these phases are foremost applied to restore myocellular homeostasis, and in particular replenish muscle glycogen reserves, before attempting to restore secondary metabolic perturbations induced by cellular functioning (Egan & Zierath, 2013). Considering this, the slightly elevated metabolic rates observed (up to 24 h post-race) could be attributed to the increased reliability on lipid oxidation as an energy source immediately post-exercise, allowing for the 24 h–48 h convalescence of muscle (thereafter hepatic) glycogen (Bielinski *et al.*, 1985; Kenttä & Hassmén, 1998; Egan & Zierath, 2013).

This phenomenon may further support the delayed recovery of lipids and associated metabolites observed by previous studies encompassing the mapping of the unaided metabolic recovery trend of athletes after eccentric exercise (Nieman *et al.*, 2013; Traiperm *et al.*, 2016; Davison *et al.*, 2018). Here, unaided recovery typically

refers to the systematic recuperation process without the assistance of any pertinent recovery aids (Section 2.5.2), thus referring to the body's natural mechanism of action to oppose exercise-induced stress. Furthermore, it has been annotated that although TBARS, TCA cycle, urea cycle, and purine/pyrimidine pathway intermediates incline towards baseline-related concentrations during the initial stages after exercise, the majority remain perturbed for at least 14 h–24 h (Lewis *et al.*, 2010; Daskalaki *et al.*, 2014; Traiperm *et al.*, 2016; Oliveira *et al.*, 2017; Davison *et al.*, 2018). However, besides the validity of this field of study, very limited literature is specifically aimed at determining the natural, unaided metabolic recovery trend of athletes within an extended period (>24 h) after ER events. Elucidation of these trends may not only improve our understanding of the adaptive metabolic capabilities of athletes, but also provide clues to more targeted, perhaps even personalised recovery approaches and strategies to expedite the recovery process.

2.5.2. Recovery modality aided post-exercise recovery

Besides the unequivocal functionality of some recovery modalities to recuperate the systematic adaptations and enhance performance, quintessential recovery aids should improve training tolerance and the athlete's systematic adaptation capacity (Allen *et al.*, 2019). To date, the most popular exercise recovery modalities investigated include physical (heat- and cryotherapy, massage therapy, stretching, and compression garments) as well as nutritional supplementation (functional food ingestion, carbohydrate loading, BCAA and protein powder supplements) interventions (Harty *et al.*, 2019; Peake, 2019). Although physical interventions have proven to be substantially beneficial in terms of structural recovery after exercise (Hill *et al.*, 2017; Allen *et al.*, 2019; Peake, 2019), research pertaining to nutrition and supplement interventions effects are reportedly more concurrent (Peake, 2019) in terms of metabolic, molecular and structural recovery. However, it should be highlighted that a substantial amount of the nutritional and supplementation aided recovery strategies investigated are based on the “long-term” (before, during and after) ingestion rather than acute post-race ingestion.

One of the nutritional strategies that have gained some scientific attention within the last decade, is the ingestion of functional foods since it is a relatively easily accessible and sustainable, cost-effective option. Although various definitions of “functional foods” exist, simply put, these are natural and/or fortified foods rich in bioactive compounds that provide an added advantage to human health, besides its calorie content (Tarabella *et al.*, 2019; Thakur *et al.*, 2020). Bioactive compounds (occasionally referred to as phytonutrients) consist of a very heterogeneous group of components (i.e. polyphenols, carotenoids, curcuminoids, anthocyanins, vitamins, etc.) which are most abundantly found in various fruits, vegetables, and whole grains (Galanakis, 2017). Whilst the potency of bioactive compounds mainly depends on its bioavailability, i.e. the measure of effective absorption from the gastrointestinal tract into the circulation, as well as the subsequent bio-accessibility and bioactivity (Galanakis, 2017; Thakur *et al.*, 2020), these compounds are still associated with numerous health benefits such as the promotion of cardiovascular health, regulation of glucose homeostasis and thus management of diabetes, prevention of DNA damage, and the impedance of cholesterol

accumulation (Galanakis, 2017; Hadipour *et al.*, 2020; Nahar *et al.*, 2020; Thakur *et al.*, 2020). Considering these effects, as well as the fact that the majority of functional fruits and vegetables possess extensive free radical scavenging, anti-inflammatory, anti-oxidant, and anti-bacterial properties (Forni *et al.*, 2019), the efficacy of some of these (cherries, pomegranates, bananas, blackcurrants, carrots, pineapple, beetroot, etc.) have been investigated as possible physiological recovery and performance-enhancing supplements (Allen *et al.*, 2019; Harty *et al.*, 2019; Peake, 2019). However, limited literature exists regarding the effect of functional foods on metabolic recovery after endurance exercise. From the select few that have attempted to fill this void, it was generally indicated that functional fruits and vegetables expedited the recovery process to some extent as its sugar/carbohydrate content (if ingested during and after exercise) not only reduced fuel substrate catabolism rates during the race, but also gave rise to gut-derived phenols that aid in immune cell function and prevent ROS production (Nieman *et al.*, 2012; Nieman *et al.*, 2015; Levers *et al.*, 2016; Nieman *et al.*, 2019; Farag *et al.*, 2020). In light of this, it has been reported that the antioxidant and anti-inflammatory capacity of beetroot (*Beta Vulgaris L.*) surpasses that of most other fruits and vegetables, most likely due to its rich betalain and polyphenol (amongst others) phytonutrient content (Hadipour *et al.*, 2020). Betalains are water-soluble pigments responsible for the vibrant colour of beetroot fibres and give rise to both the violet (betacyanins) and yellow (betaxanthins) pigments (Rodriguez-Amaya, 2019). Besides the electron-donating abilities aiding in ROS scavenging, betalains (and betaines) are further associated with an almost endless list of health benefits including reduced cholesterol accumulation, elevated HDL production, disruption of cyclooxygenase and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) functionality, suppression of TNF- α and IL-6, prevention of lipid peroxidation, etc. (Hadipour *et al.*, 2020; Mirmiran *et al.*, 2020). Beetroot is also renowned for its high nitrate content, which may be beneficial in enhancing the nitrogen availability in the body via the nitrate-nitrite-NO pathway (Clifford *et al.*, 2017; Dominguez *et al.*, 2018). Briefly, upon ingestion, the nitrate content of beetroot fibres is reduced to salivary nitrite, by facultative anaerobic bacterial species that reside in the oral cavity. Once salivary nitrite meets the acidic environment of the stomach, it is further reduced to nitrous acid and subsequently NO, which can later be absorbed in the gastrointestinal tract (Lundberg *et al.*, 2008; Clifford *et al.*, 2017; Dominguez *et al.*, 2018; Amdahl *et al.*, 2019). Additionally, residual and/or dietary nitrite may also be directly absorbed by the gastrointestinal tract, whereupon it is later reduced to NO by various haem-proteins and molybdopterin present in the plasma (Amdahl *et al.*, 2019). Once in the circulatory system, NO not only aids in vasodilation, but it also promotes mitochondrial biogenesis, tubular gas exchange, and reduces renovascular resistance (Mirmiran *et al.*, 2020).

Considering all of these potentially beneficial effects of beetroot consumption, it is not surprising that the Australian Institute of Sport (2019) classified this vegetable as a Class A performance-enhancing (supported by adequate scientific research) supplement. However, studies opposing this notion, thus indicating no added advantage of beetroot in terms of performance after exercise, are steadily gaining momentum (Clifford *et al.*, 2017; Balsalobre-Fernandez *et al.*, 2018; Garnacho-Castano *et al.*, 2018). This may stem from the relatively low bioavailability of polyphenols when in a glycated form, growth imbalances of the gut microbiome (GM),

as well as inadequate doses and/or ingestion frequency (Betteridge *et al.*, 2016; Brglez Mojzer *et al.*, 2016; Olsson *et al.*, 2019). Nevertheless, besides all the effects of beetroot, in particular, the promotion of mitochondrial biogenesis, vasodilation, as well as anti-inflammatory and antioxidant properties, it is reported that beetroot has a substantial regulatory impact on glucose and insulin metabolism, which may increase insulin sensitivity and secretion. These properties would be substantially beneficial to metabolic recovery after ER events, considering the effects (Sections 2.2–2.3) induced. As such, it may be worth investigating the feasibility of this vegetable, or a supplement derived from such, as a possible metabolic recovery agent.

2.6. REFERENCES

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CHAPTER 3: METHODOLOGY AND MATERIALS

3.1. INTRODUCTION

Selecting the appropriate sample matrix is a key component of any metabolomics experimental procedure (Stevens *et al.*, 2019), and typically depends on numerous considerations, such as the biological question, type of metabolites of interest, as well as the analytical technique selected. Since blood suffuses every organ in the human body, it serves as a liquid carrier for all metabolites excreted/produced in response to a physiological or pathological stimulus (Psychogios *et al.*, 2011). Considering this, as well as its readily available nature, it is not surprising that blood is one of the preferred matrices utilised in metabolomics investigations (Liu *et al.*, 2018), especially in ER-based metabolomics studies, as described in Chapter 2 (Table 2-2). The liquid portion of blood typically consists of two components viz. plasma and serum, which differs based on whole blood preparation methods. Serum is the fraction that remains after clotting and centrifugation of whole blood samples, resulting in a clear liquid, free of fibrous proteins, blood cells, and clotting factors (Smith *et al.*, 2020). Whilst studies have indicated some distinctions between serum and plasma metabolic fingerprinting profiles (Liu *et al.*, 2018), serum generally contains higher metabolite concentrations, thus suggesting an overall higher sensitivity during biomarker discovery (Paglia *et al.*, 2018). Nevertheless, the analytical popularity (compared to others such as tissue biopsies, cerebral spinal fluid, and sputum) of these fractions stems from the fact that these are not only readily available and relatively stable in terms of customary human confounders (age, sex, etc.), but this biofluid also requires minimal preservation and processing for metabolite identification (Serkova *et al.*, 2011). However, as in the case of most biological matrices, blood as a biofluid may be associated with some limitations including semi-invasive sampling (Beale *et al.*, 2016), pre-processing procedures (Smith *et al.*, 2020), and the possibility that the collection tubes may release additional compounds into the sample matrix (Liu *et al.*, 2018). Furthermore, trypanophobia (a fear of needles) may lead to anxiety in the subjects, which may potentially contribute to metabolic stress after a perturbation (Du Preez *et al.*, 2017). Whilst these limitations have to be considered upon interpreting the results of this investigation, it is clear that serum as a formidable biological matrix to use as a means of addressing the aims of this investigation.

3.2. EXPERIMENTAL DESIGN

Considering that this Ph.D. investigation forms part of a larger collaboration project, all the participant recruitment, interventions and sample collection, were performed by the Northumbria University (Newcastle upon Tyne, UK), whereas the NWU was responsible for further characterisation of the samples by employing an untargeted GCxGC-TOFMS metabolomics research approach. A detailed outline of the overall metabolomics experimental design employed to address the aims of this investigation is presented in Figure 3-1, and the relevant sections discussed below. In summary, blood samples of 31 athletes (19 males and 12 females) were collected (antecubital venesection) 24 h before (pre-marathon) and immediately after (within 30 min post-marathon), as well as 24 h and 48 h after completing the Druridge Bay Marathon (Northumberland, UK) (Clifford *et al.*, 2017). During the two consecutive days following the race, athletes

received either beetroot juice (n=15 athletes; 9 males and 6 females) or placebo (n=16 athletes; 10 males and 6 females) supplements. Hence, a total of 124 serum samples were collected for analysis, which was divided into smaller batches (n=2) for analysis. Each batch represented randomly selected samples over the entire sample set and included five quality control (QC) samples (Section 3.2.5). An in-house total metabolome extraction method was used to extract the samples, followed by derivatisation (Section 3.2.6), and analysis on a GCxGC-TOFMS (Section 3.2.7). QC samples were used in a quality assurance step during data processing to assess the validity of the results obtained and to correct for possible batch effects of the results generated, as indicated in red in Figure 3-1. The data generated from these analytical procedures were processed using ChromaTOF software (Section 3.2.7), whereafter contaminants and column-related metabolites were removed from the data matrix. Hereafter, the data were subjected to various statistical methods (Section 3.2.8) as a means of selecting the metabolite markers pertinent to the aims of this investigation. A further simplified representation of the relevant samples used per aim is presented in Figure A-1 (see Annexure A).

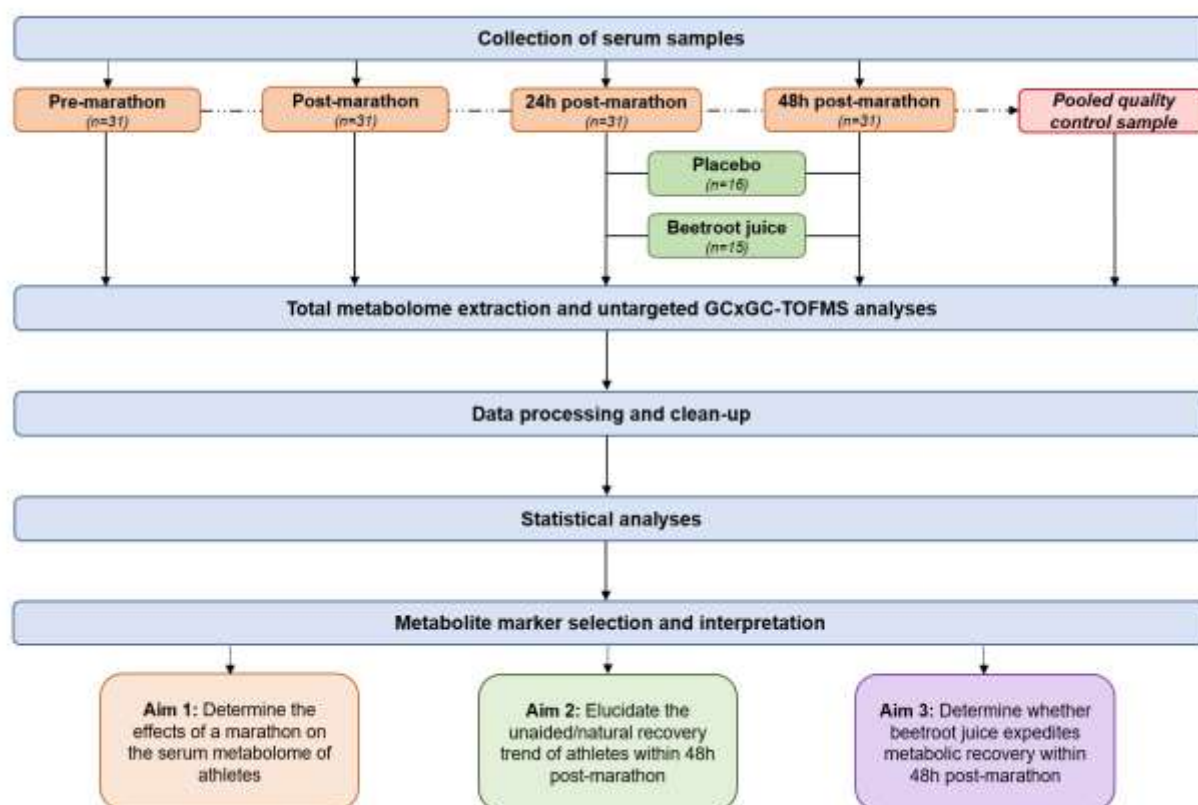


Figure 3-1: Schematic representation of the overall experimental design used to address the aims and objectives of this metabolomics investigation.

3.2.1. Ethical approval

Ethics approval for the larger scope of the study, conducted according to the Declaration of Helsinki and International Conference on Harmonisation Guidelines, was obtained from the Research Ethics Committee of

the Faculty of Health and Life Sciences at the Northumbria University in Newcastle upon Tyne, United Kingdom (reference number: HLSTC120716).

3.2.2. Participants

Participation in this study was completely voluntary, and all the participants gave written and informed consent before the commencement of any analyses (Clifford *et al.*, 2017). Prior to the marathon, all participants were required to complete a health and dietary questionnaire (with an additional menstrual cycle questionnaire for female participants), of which individuals receiving or using any anti-inflammatory treatments, chronic medication, as well as those with any food allergies, cardiovascular complications or musculoskeletal disorders and injuries, were excluded from the study. Considering the ethical implications concerning ethnicity classifications of participants, when it is not central to the research topic, and the fact that this study was not biased towards any ethnic groups, nor were any of the aims a comparison of ethnicity with respect to the marathon-induced changes to the human metabolome, athlete ethnicity was not recorded. Furthermore, all athletes were instructed to refrain from exercising and/or using any alternative recovery modalities (heat therapy, cryotherapy, inflammatory drugs, antioxidant vitamins, compression garments, etc.) during the recovery period of this investigation. Withal, the use of anti-bacterial mouth wash was also prohibited as a means of conserving the proposed bacterial nitrate-nitrite conversion of beetroot nitrate in the oral cavity. Lastly, athletes' dietary intake was recorded throughout the investigation in attempt to compensate for confounding variation that may affect results. An overview of the participant characteristics are presented in Table 3-1.

Table 3-1: A summary of the participant characteristics of the placebo and beetroot ingesting cohorts.

Participant demographical information	Placebo cohort (n=16; 10 males and 6 females)	Beetroot cohort (n=15; 9 males and 6 females)
	Average $\pm$ standard deviation	
Age (years)	39 $\pm$ 12	42 $\pm$ 10
Pre-marathon athlete weight (kg)	72.2 $\pm$ 11.9	70.3 $\pm$ 7.9
Post-marathon athlete weight (kg)	70.8 $\pm$ 11.7	69 $\pm$ 7.4
Marathon experience (years)	8 $\pm$ 7	11 $\pm$ 10
Marathon experience (races)	21 $\pm$ 40	10 $\pm$ 12
Finishing time (hh:mm:ss)	04:30:25 $\pm$ 00:36:48	04:07:08 $\pm$ 00:39:16

3.2.3. The Druridge Bay Marathon and supplements

The Druridge Bay Marathon promptly started at 09:00 and consisted of four laps around the Country Park (Northumberland Coast, UK), which mainly consists of grassy, concrete mixed terrain. Furthermore, approximately 1.6 km of each lap consisted of soft sand (± 6.4 km) (Clifford *et al.*, 2017) and may contribute to the slower finishing times (Table 3-1). Temperature, wind speed and humidity readings were obtained in

the beginning and the end of the race (13:00–14:00), and only indicated a slight elevation in wind speed and air temperature toward the end of the race (Clifford *et al.*, 2017). Immediately following the race (after post-marathon venesection; Section 3.2.4) athletes ingested a series of either beetroot juice or isocaloric placebo (maltodextrin, protein powder, and fruit squash mixture) supplements. These supplements were indistinguishable in appearance and were consumed as follows: 3x 250 mL supplements on the day of the marathon (immediately after the post-marathon sampling, ± 3 h post-race, and at 20:00 respectively), 3x 250 mL supplements on the first day after the marathon (upon waking-up, with lunch, and with supper respectively), and 1x 250 mL supplement upon waking on the second day post-marathon. Table 3-2 provides a summary of the nutrient composition of these respective supplements.

Table 3-2: Macro-nutrient composition of the beetroot juice and placebo supplements provided to athletes [adapted from Clifford *et al.* (2017)].

Macro-nutrient composition	<u>Supplements</u>	
	Beetroot	Placebo
	(Per 250 mL serving)	
Energy (kcal)	81.0	76.8
Glycaemic content (g)	16.4	16.4
Fats (g)	0.4	Trace amounts
Protein (g)	2.8	2.8

3.2.4. Clinical sample collection and storage

As indicated in Figure 3-1, blood samples of the athletes were obtained (using antecubital fossa venesection of the basilica vein) at four time-points including 24 h pre-marathon, immediately post-marathon, as well as 24 h and 48 h post-marathon, of which the baseline and recovery samples were obtained in a laboratory setting. The participants were required to be in a hydrated, yet fasted state (for a minimum of two hours) at the time of baseline (pre-marathon) venesection, which was performed between 10:00 and 18:00 on the day preceding the race, in attempt to reduce additional metabolic changes induced by the venesection stress, as well as to limit interference to the athletes' pre-marathon regimens. Hereafter, post-marathon samples were obtained within 30 min after completing the race, thus dictating the approximate times of the 24 h and 48 h post-marathon sample collection on the consecutive days following the marathon. Upon venesection, samples were collected in standard 10 mL vacutainer vials, placed on ice, and transported to the Faculty of Health and Life Sciences, Department of Sport, Exercise and Rehabilitation Laboratory at the Northumbria University. Here, samples were left to clot (at room temperature) for 30 min before being centrifuged at 3 000 *g* for 10 min. The serum portion (supernatant) was then extracted, immediately frozen (-80°C), and transported (on dry ice) to the NWU, Human Metabolomics: Laboratory of Infectious and Acquired Diseases for further metabolomics analyses. All samples were stored at -80°C until metabolomics analysis commenced.

3.2.5. Quality control samples

QC samples ensure the validity/reliability of the data obtained since it is used as an integral factor in determining and correcting for potential batch effects (i.e. analytical drifts) that may occur. A single pooled QC sample was compiled using 50 μL of each serum sample, which was further divided into five smaller (50 μL) QC aliquots that were extracted and derivatised with the actual athlete samples. Although these QC samples were injected after approximately every seventh sample during analysis, these were also consecutively ($n=3$) injected into the apparatus at the beginning and the end of each batch, as part of the machine calibration protocol.

3.2.6. Total metabolome extraction and derivatisation

All of the participant serum samples (including pooled QC samples) were subjected to an in-house total metabolome extraction (SOP number: HM-MET-056). During this process, smaller aliquots of the samples (50 μL) were prepared, before adding 50 μL of the internal standard (3-phenylbutyric acid; 0.45 $\mu\text{g/mL}$) dissolved in a chloroform:methanol:water (1:3:1) solution. The latter serves as a metabolite conservation step to ensure that larger polar and non-polar compounds and associated metabolites (e.g. AAs, FAs, purines, pyrimidines, alkenes, alkanes, etc.) can be analysed using the GCxGC-TOFMS. Protein precipitation of the samples commenced by adding 300 μL of ice-cold acetonitrile, whereafter each vial was subjected to a 2 min mixing step (REAX D-91126 vortex; Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) and 10 min centrifugation at 4000 rpm. The supernatant was then extracted, transferred to a clean GC-MS vial, and dried with a light stream of nitrogen gas for 45 min, whilst being housed in a heating block set at 40°C. Once the solvent evaporated, 25 μL of methoxamine hydrochloride dissolved in pyridine (15 mg/mL) was added to each sample and incubated for 90 min at 50°C. Lastly, 40 μL of BSTFA enriched with 1% TCMS was added, and samples were left to derivatise/silylate for 60 min at 60°C. The derivatised samples were then transferred to a clean glass GC-MS vial containing a vial-insert before being analysed.

3.2.7. GCxGC-TOFMS analysis and data processing

These derivatised samples were injected into a Pegasus 4D GCxGC-TOFMS analytical apparatus (LECO Africa (Pty) Ltd, Johannesburg, South Africa) in a randomised order using a Gerstel 104 auto-sampler tray (GmbH and co. KG, Mülheim van der Ruhr, Germany), with QC samples preceding approximately every 7th sample for quality assurance purposes (Section 3.2.5). Each sample (1 μL) was injected using a 1:3 split ratio, with a constant injector temperature of 270°C and a carrier gas (purified helium) flow rate of 1 mL/min. The primary oven, equipped with a Restek Rxi-5MS capillary column (30 m; 0.25 μm diameter and 0.25 μm film thickness), was set at an initial temperature of 70°C before incrementally increasing (4°C/min) to a final temperature of 300°C (maintained for 2 min). In addition, the secondary oven, fitted with a Restek Rxi-17 capillary column (1 m; 0.25 μm diameter and 0.25 μm film thickness), was programmed with an initial temperature of 85°C, which was set to incrementally increase with 4.5°C/min until a final temperature of 300°C was reached and maintained for 2 min. The thermal modulator, amid the two columns, was set to intermittently

pulse streams of hot and cold nitrogen gas for approximately 0.5 s (every 3 s). Mass spectra (ms) of ions (50–800 m/z range) was acquired at a rate of 200 ms/s, while the ms of the first 400 s of the run was discarded to exclude extraction solvent elution. However, the latter was not removed from the time-axis of the primary column to ensure compound retention time validity. The transfer line and ion source were set to function at 270°C and 220°C, respectively, with a detector voltage of 1600 V and ion filament bias of -70e V.

After analytical analysis, the data generated was processed using the ChromaTOF software (LECO Corporation), which included ms deconvolution, peak alignment and peak annotation via mass fragment pattern and retention time comparisons with in-house libraries compiled from previously injected standards (Luies & Loots, 2016). ChromaTof parameter settings included peak widths of 9 and 0.2 for the 1st and 2nd dimension respectively, and a signal-to-noise ratio of 100, with a maximum of 100 000 unknown peaks allowed (to remove bias). All metabolites with molecular masses between 50–800 Da were detected with a minimum of 70% similarity match to the in-house libraries for identification purposes. The experimental procedure performed was in accordance with proposed guidelines and regulations, as approved by the NWU and forms part of the laboratory's standard operating procedures (SOP number: HM-MET-056).

3.2.8. Data clean-up, quality assurance, and statistical analyses

As is standard practice during all metabolomics studies, the data was subjected to several “clean-up steps” such as the removal of column and/or reagent-related compounds, as well as the merging of duplicate peaks of specific compounds (AAs, sugars, etc. known to elute at different time-point). Hereafter, all the compounds in the dataset were normalised relative to the internal standard and further subjected to various clean-up steps. These mainly include: (1) a 50% zero value filter which removes compounds that are not present in >50% of both groups (Luies & Loots, 2016); (2) zero value replacement with either half of the detection limit value (smallest value in the dataset) or random, small values along the tail distribution of the data (Fernandez *et al.*, 2000; Luies & Loots, 2016); (3) a QC coefficient of variation (CV) filter that removes compounds with CV values higher than the selected parameter (Chaleckis *et al.*, 2016); (4) drift or batch correction of compounds based on QC sample repeats (Wang *et al.*, 2013); as well as (5) log transformation; and (6) auto-scaling (Feng *et al.*, 2014). Considering that a few of the aforementioned clean-up steps depend on the QC samples, a principal component analysis (PCA) scores plot (Figure 3-2a) was constructed to visualise the variance of QCs relative to the samples. In this figure, it is clear that three of the QC samples do not conform with the confined, clustered nature of the other QC aliquots relative to the samples. Further investigation identified these to be the last three injected QC samples of Batch 2 and was attributed to possible sample evaporation resulting from multiple previous reinjections. After the removal of these, the QC aliquots and samples were inherently clustered (Figure 3-2b), suggesting the absence of a batch effect.

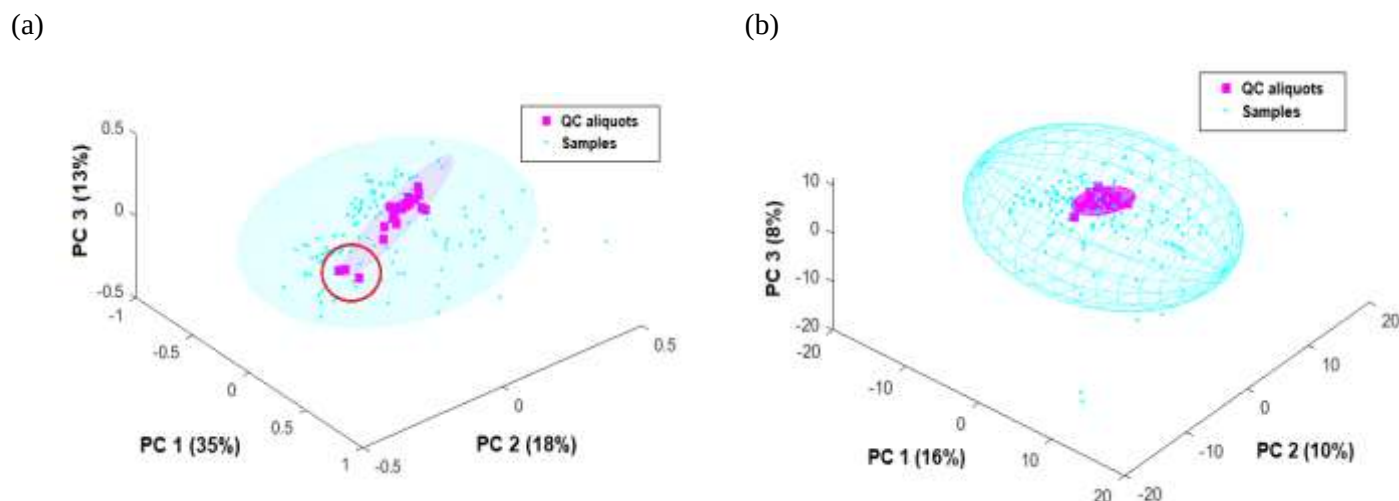


Figure 3-2: Principal component analysis scores plot of the obtained serum samples (a) before and (b) after removal of the outlier quality control samples. Abbreviation: PC: Principal component.

Following data clean-up, various multivariate and univariate statistical approaches were employed to identify the metabolites that are significant pertaining to each aim. As such, each of the following Chapter consists of different statistical designs and selection models and is therefore discussed in the relevant statistical section of each Chapter (Chapters 4–6).

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CHAPTER 4: MARATHON-INDUCED METABOLIC ADAPTATIONS

This chapter has been published in an international peer-reviewed journal:

Stander, Z., Luies, L., Mienie, L.J., Keane, K.M., Howatson, G., Clifford, T., Stevenson, E.J. & Loots, D.T. 2018. The altered human serum metabolome induced by a marathon. *Metabolomics*, 14(150). Impact factor: 3.16

4.1. ABSTRACT

Endurance races have been associated with a substantial amount of adverse effects which could lead to chronic disease and long-term performance impairment. However, little is known about the holistic metabolic changes occurring within the serum metabolome of athletes after the completion of a marathon. Considering this, the aim of this study was to better characterise the acute metabolic changes induced by a marathon. Using an untargeted two-dimensional GCxGC-TOFMS mass spectrometry metabolomics approach, pre- and post-marathon serum samples of 31 athletes were analysed and compared to identify those metabolites varying the most after the marathon perturbation. PCA of the comparative groups indicated natural differentiation due to variation in the total metabolite profiles. Elevated concentrations of carbohydrates, FAs, TCA cycle intermediates, ketones and reduced concentrations of AAs indicated a metabolic shift between various fuel substrate systems. Additionally, elevated OCFA and α -hydroxy acids indicated the utilisation of α -oxidation and autophagy as alternative energy-producing mechanisms. Adaptations in gut microbe-associated markers were also observed and correlated with the metabolic flexibility of the athlete. From these results, it is evident that a marathon places immense strain on the energy-producing pathways of the athlete, leading to extensive protein degradation, oxidative stress, mTORC I inhibition and autophagy. A better understanding of this metabolic shift could provide new insights for optimising athletic performance, developing more efficient nutrition regimens and identify strategies to improve recovery.

4.2. INTRODUCTION

Although physical activity has been shown to be substantially beneficial to human health (Ojiambo, 2013), various negative effects including cardiovascular dysfunction (Webner *et al.*, 2012), muscle damage (Howatson *et al.*, 2010), increased propensity to upper respiratory tract infection (Robson-Ansley *et al.*, 2012) and severe inflammation (Bonasia *et al.*, 2015) have been associated with running extensive distances, such as that of endurance races. Even though these races have become increasingly popular, limited research is based on the elucidation of the effects of these races using a metabolomics approach. Metabolomics is defined as the identification and quantification of the small metabolite compounds (<1500 Da) present in a biological system in order to determine the physiological effects induced by a specific perturbation (Heaney *et al.*, 2017). Since metabolites are typically the end-products of the genome, transcriptome and proteome, alterations in these are indicative of the overall physiological state of the investigated biological system (Heaney *et al.*, 2017).

Previous metabolomics studies have indicated elevated concentrations of various carbohydrate/glycolysis metabolite intermediates, indicative of free glucose utilisation as the preferred energy source during strenuous physical activity (Lewis *et al.*, 2010; Salway, 2012; Waśkiewicz *et al.*, 2012). Furthermore, significant alterations to the TCA cycle intermediates were induced by a marathon (Turer *et al.*, 2014) and could be attributed to the additional strain placed on the ETC, causing an imbalanced NADH:NAD⁺ ratio (Esterhuizen *et al.*, 2017). According to previous work (Stellingwerff, 2012), free glucose and other carbohydrate stores can become depleted within approximately 90 min after the start of the race, which most likely lead to the utilisation of alternative fuel substrates (lipids and AAs) for energy production (Waśkiewicz *et al.*, 2012). Increased lipolysis activity results in elevated serum glycerol and free FAs (Lewis *et al.*, 2010; Waśkiewicz *et al.*, 2012), the latter of which produce acetyl-CoA via β -oxidation and subsequent energy via the TCA cycle and ETC (Salway, 2012). Furthermore, the increased synthesis of acetyl-CoA could also ascribe the elevated ketone body concentrations previously reported (Pechlivanis *et al.*, 2010), as it is a key component of ketogenesis. In the event that the strenuous physical activity continues beyond the capacity of the athlete's lipid stores, or if the traditional lipid oxidation pathways become saturated (Staron *et al.*, 1989), the athlete's metabolism shifts towards protein catabolism (resulting in reduction blood AA levels) in an attempt to synthesise the energy required to complete the marathon (Lewis *et al.*, 2010). These AAs are primarily oxidised to pyruvate and acetyl-CoA, both of which can serve as TCA cycle influx substrates for energy production (Salway, 2012). Additionally, protein degradation has been shown to alter purine catabolism, resulting in elevated AMP, inosine-monophosphate, hypoxanthine, xanthine, urate and allantoin (Turer *et al.*, 2014), the latter of which is a urate derivative and a surrogate index of oxidative stress (Lewis *et al.*, 2010).

Although these metabolomics studies provide some clues to the metabolic alterations that occur during strenuous physical activity such as long-distance endurance races, very few of these employed an untargeted metabolomics approach. Considering this, an untargeted GCxGC-TOFMS metabolomics approach was used

to holistically compare the serum metabolite profiles of 31 recreational marathon athletes before and after the completion of a marathon (42 km), to better characterise the acute metabolic changes induced by exercise stress.

4.3. MATERIALS AND METHODS

4.3.1. Participants and clinical samples

Considering that this section is aimed at determining the effects of a marathon on the serum metabolome, only pre-marathon and immediately post-marathon serum samples of the 31 athletes (19 males and 12 females) were included in this part of the investigation. A detailed description of the marathon layout (Section 3.2.3), participant selection and/or eligibility criteria (Section 3.2.2), as well as sample collection (Section 3.2.4), is provided in Chapter 3 of this thesis. Athlete participation in this investigation was completely voluntary, and all the participants gave written and informed consent before analysis commenced. Although Table 3-1 (Chapter 3) depicts the characteristics of all the participants based on supplement categorisation, Table 4-1 provides a summary of the overall demographic information of all athletes included in this section of the investigation.

Table 4-1: Summary of the participant demographic information.

Participant demographical information	Average $\pm$ standard deviation
Age (years)	41 $\pm$ 12
Pre-marathon athlete weight (kg)	71.3 $\pm$ 10.1
Post-marathon athlete weight (kg)	69.2 $\pm$ 9.7
Marathon experience (years)	9 $\pm$ 8
Marathon experience (races)	16 $\pm$ 29
Finishing time (hh:mm:ss)	04:19:09 $\pm$ 00:49:01

4.3.2. Sample analysis

After sample collection (Section 3.2.4), the frozen serum samples were transported to the NWU for further characterisation. Here, samples were subjected to a total metabolome extraction and derivatisation (Section 3.2.6), before being analysed using the 4D Pegasus GCxGC-TOFMS (Section 3.2.7). The resulting data was then processed by the ChromaTOF software, which subsequently utilises the ms of previously injected standards to identify the compounds present in the analysed samples (Section 3.2.7).

4.3.3. Data clean-up and statistical analyses

The cleaned data was then normalised in relation to the internal standard and subjected to several clean-up steps including a 50% zero filter, QC drift correction, 30% QC-CV filter (removing compound that have CV>30%) and zero value replacement (Fernandez *et al.*, 2000; Luies & Loots, 2016). Hereafter, a natural shifted log transformation was performed to correct for skewed variable distribution, as well as auto-scaling to align all variables (Van den Berg *et al.*, 2006). Furthermore, pre-marathon and post-marathon serum metabolite profiles of all 31 marathon athletes were compared by employing both multivariate and univariate statistical models (MATLAB software (2012), adjusted with the PLS toolbox (2016)). Multivariate approaches included a multi-level (ML)- PCA to determine whether a natural differentiation occurred between the comparative groups, and partial least square–discriminant analysis (PLS-DA) to characterise the group separation (Luies & Loots, 2016). The univariate data analyses included a Wilcoxon signed-rank test, corrected for multiple testing by the Benjamini–Hochberg procedure, to assess the statistical significance of each compound (Benjamini & Hochberg, 1995; Rosner *et al.*, 2006), while an independent effect size calculation (Wilcoxon signed effect size) was used to assess the practical significance of each detected metabolite (Tomczak & Tomczak, 2014).

4.4. RESULTS

The PCA differentiation between serum metabolite profiles (Figure 4-1) of the marathon athletes before and after the completion marathon was clearly defined. The total amount of variance explained by the first three principal components (R^2X cum) was 53%, of which PC1 accounted for 24%, PC2 for 20% and PC3 for 9%. Additionally, the PLS-DA model (results not shown) showed a modelling parameter R^2Y (cum) of 48.81%, indicating the total variance of the response Y, and a Q^2 (cum) of 83.37%, indicating the cross-validation variation due to the response Y.

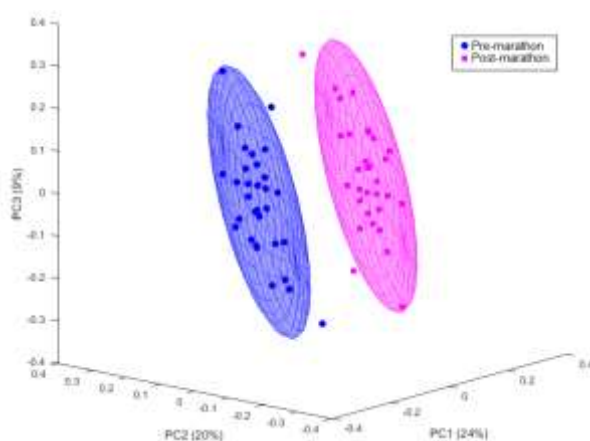


Figure 4-1: Multilevel principal component analysis scores plot showing clear differentiation of the serum samples of marathon athletes before (denoted by circles) and after (denoted by squares) the completion of a marathon. The variance accounted for are indicated in parenthesis. Abbreviations: PC: principal component.

Since the aim of this study was to attain a holistic view of the altered human serum metabolome induced by a marathon, compounds with a PCA power value ≥ 0.5 or Wilcoxon p -value ≤ 0.017 (BH-critical value) or an effect size ω -value ≥ 0.3 were considered significant and interpreted based on their associated metabolic/biochemical pathways. This multi-statistical approach yielded an initial list of 78 metabolite markers, of which 70 metabolite markers could be annotated by comparison of their mass spectra and retention times to that of commercially available and in-house libraries developed from previously injected standards (see Table 4-2).

Table 4-2: The significant serum metabolite markers best describing the variation between the pre- and post-marathon groups, listed alphabetically.

Metabolite name (PubChem ID)	Pre-marathon athletes: Concentration ($\mu\text{g/mL}$) (standard deviation)	Post-marathon athletes: Concentration ($\mu\text{g/mL}$) (standard deviation)	Wilcoxon Signed Rank test (p -value)	(ω -value)	PCA (Power)
α -Aminomalonate (100714)	0.046 (0.022)	0.025 (0.009)	3.1×10^{-4}	0.458	0.031
α -Ethylhydracrylate (188979)	0.002 (0.001)	0.005 (0.002)	1.3×10^{-6}	0.615	0.025
α -Hydroxybutyrate (11266)	0.019 (0.011)	0.045 (0.023)	2.8×10^{-6}	0.595	0.018
α -Hydroxyoctanoate (94180)	8.7×10^{-5} (5.3×10^{-5})	1.2×10^{-4} (6.3×10^{-5})	0.001	0.413	0.003
α -Ketoglutarate (51)	2.9×10^{-4} (2.1×10^{-4})	4.5×10^{-4} (1.8×10^{-4})	1.1×10^{-4}	0.490	0.009
α -Ketoisovalerate (5204641)	0.002 (0.001)	0.003 (0.001)	1.7×10^{-4}	0.478	0.017
α -Keto- β -methylvalerate (47)	0.009 (0.003)	0.007 (0.002)	0.005	0.358	0.025
α -Linolenate (5280934)	1.6×10^{-4} (8.1×10^{-5})	4.5×10^{-4} (2.6×10^{-4})	2.1×10^{-6}	0.602	0.021
β -Hydroxybutyrate (441)	0.021 (0.019)	0.215 (0.161)	1.2×10^{-6}	0.617	0.030
β -Hydroxyhexanoate (151492)	3.2×10^{-4} (1.6×10^{-5})	8.9×10^{-4} (3.1×10^{-4})	2.1×10^{-6}	0.602	0.033
β -Hydroxyisobutyrate (87)	2.9×10^{-5} (4.9×10^{-5})	2.9×10^{-4} (1.4×10^{-4})	1.2×10^{-6}	0.617	0.034
β -Hydroxyisovalerate (69362)	0.002 (0.001)	0.002 (0.001)	0.011	0.321	0.009
β -Hydroxypropionate (68152)	0.003 (0.001)	0.004 (0.001)	0.007	0.343	0.013
β -Hydroxy- α,β -didehydrosebacate (5366445)	0.005 (0.002)	0.006 (0.002)	0.009	0.333	0.013
5-Dodecenoate (5312377)	1.3×10^{-4} (2.8×10^{-5})	0.002 (0.001)	1.2×10^{-6}	0.617	0.037
5-Pregnene-3 β ,20 α -diol (312224064)	3.0×10^{-4} (2.7×10^{-4})	6.1×10^{-4} (4.3×10^{-4})	3.4×10^{-6}	0.590	0.007
10-Heptadecenoate (86289714)	0.001 (0.001)	0.003 (0.001)	1.2×10^{-6}	0.617	0.030
11-Eicosenoate (142770)	0.001 (0.001)	0.004 (0.001)	1.3×10^{-6}	0.615	0.033
11,14-Eicosadienoate (3208)	0.002 (0.001)	0.003 (0.001)	9.3×10^{-4}	0.421	0.008
Acetoacetate (96)	7.5×10^{-5} (5.4×10^{-5})	2.1×10^{-4} (1.5×10^{-4})	0.002	0.393	0.014

Alanine (5950)	4.2x10 ⁻⁴ (2.9x10 ⁻⁴)	2.6x10 ⁻⁴ (1.5x10 ⁻⁴)	0.004	0.368	0.003
Arabitol (439255)	0.004 (0.001)	0.005 (0.002)	9.9x10 ⁻⁴	0.418	0.006
Aspartate (5960)	0.020 (0.012)	0.013 (0.007)	0.014	0.311	0.019
Citrate (311)	9.6x10 ⁻⁴ (4.5x10 ⁻⁴)	0.002 (0.001)	8.9x10 ⁻⁵	0.498	0.012
Dimethylglycine (673)	0.023 (0.013)	0.017 (0.008)	0.011	0.324	0.008
D-Rhamnose (5460029)	0.001 (0.001)	0.001 (0.001)	1.1x10 ⁻²	0.324	0.006
Erythritol (222285)	0.007 (0.002)	0.012 (0.004)	1.6x10 ⁻⁶	0.610	0.016
Ethyl- α -D-glucopyranoside (91733361)	0.169 (0.085)	0.213 (0.094)	0.008	0.336	0.003
Fumarate (444972)	0.005 (0.002)	0.007 (0.002)	7.5x10 ⁻⁴	0.428	0.018
Glucarate (33037)	4.1x10 ⁻⁴ (1.8x10 ⁻⁴)	0.001 (0.001)	0.002	0.391	0.010
Glucose (5793)	0.001 (0.001)	0.003 (0.005)	8.7x10 ⁻⁶	0.565	0.025
Glutarate (743)	3.1x10 ⁻⁴ (1.9x10 ⁻⁴)	4.7x10 ⁻⁴ (2.8x10 ⁻⁴)	0.009	0.331	0.009
Glycerate (752)	0.005 (0.002)	0.008 (0.003)	6.9x10 ⁻⁵	0.505	0.015
Glycerol (753)	0.088 (0.042)	0.447 (0.143)	1.2x10 ⁻⁶	0.617	0.041
Glycine (750)	0.042 (0.019)	0.026 (0.013)	7.5x10 ⁻⁴	0.428	0.024
Heptadecanoate (10465)	0.004 (0.001)	0.006 (0.001)	1.2x10 ⁻⁶	0.617	0.020
Ibuprofen (3672)	7.5x10 ⁻⁶ (1.8x10 ⁻⁵)	0.004 (0.013)	6.4x10 ⁻⁵	0.508	0.011
Indole-3-propionate (3744)	0.002 (0.001)	0.001 (0.001)	1.8x10 ⁻⁴	0.475	0.004
Indole-3-acetate (802)	0.003 (0.002)	0.002 (0.001)	8.1x10 ⁻⁴	0.426	0.021
Laurate (3893)	0.011 (0.003)	0.020 (0.005)	1.2x10 ⁻⁶	0.617	0.026
Leucine (6106)	0.130 (0.087)	0.064 (0.044)	0.002	0.398	0.022
Linoleate (5280450)	0.124 (0.050)	0.168 (0.055)	4.9x10 ⁻⁴	0.443	0.006
Malate (525)	0.004 (0.002)	0.007 (0.003)	3.1x10 ⁻⁶	0.592	0.018
Malonate (867)	3.1x10 ⁻⁴ (1.2x10 ⁻⁴)	4.1x10 ⁻⁴ (1.7x10 ⁻⁴)	0.005	0.353	0.006
Mannitol (6251)	0.004 (0.004)	0.007 (0.010)	0.006	0.348	0.004
Mannose (18950)	3.2x10 ⁻⁴ (4.3x10 ⁻⁴)	0.003 (0.005)	5.5x10 ⁻⁶	0.577	0.024
Methionine (6137)	0.006 (0.004)	0.003 (0.002)	6.9x10 ⁻⁴	0.431	0.017
Myo-inositol (892)	0.031 (0.030)	0.045 (0.035)	8.1x10 ⁻⁴	0.426	0.011
Monopalmitin (14900)	3.9x10 ⁻⁴ (2.1x10 ⁻⁴)	6.7x10 ⁻⁴ (3.6x10 ⁻⁴)	0.002	0.401	0.011
Myristoleate (5281119)	0.001 (0.001)	0.006 (0.002)	1.2x10 ⁻⁶	0.617	0.037
Oleate (445639)	0.127 (0.072)	0.511 (0.196)	1.3x10 ⁻⁶	0.615	0.022
Palmitate (985)	0.423 (0.124)	0.633 (0.128)	1.6x10 ⁻⁵	0.548	0.016
Palmitoleate (445638)	0.009 (0.008)	0.042 (0.022)	7.2x10 ⁻⁶	0.570	0.026

Pentadecanoate (13849)	0.004 (0.002)	0.008 (0.002)	1.2x10 ⁻⁶	0.617	0.022
Phenylalanine (6140)	0.030 (0.015)	0.020 (0.009)	0.002	0.393	0.021
<i>p</i> -Hydroxyphenylacetate (127)	0.002 (0.001)	0.003 (0.002)	0.001	0.408	0.008
<i>p</i> -Hydroxyphenyllactate (9378)	0.003 (0.002)	0.005 (0.002)	2.9x10 ⁻⁴	0.460	0.014
Pyroglutamate (7405)	0.060 (0.014)	0.050 (0.023)	2.3x10 ⁻⁴	0.468	0.009
Pyruvate (1060)	0.013 (0.009)	0.024 (0.012)	7.5x10 ⁻⁴	0.428	0.016
Serine (5951)	0.033 (0.024)	0.018 (0.011)	0.014	0.314	0.021
Sorbose (439192)	0.015 (0.006)	0.022(0.01)	6.1 x10 ⁻⁴	0.436	0.008
Squalene (638072)	0.002 (0.001)	0.003 (0.004)	0.006	0.351	0.011
Succinate (1110)	0.011 (0.008)	0.016 (0.010)	5.5x10 ⁻⁶	0.577	0.006
Tagatofuranose (12306016)	0.101 (0.058)	0.190 (0.122)	3.9x10 ⁻⁴	0.450	0.012
Talofuranose (15560229)	0.147 (0.050)	0.253 (0.090)	2.3x10 ⁻⁵	0.538	0.011
Threonate (5460407)	0.011 (0.004)	0.016 (0.005)	1.1x10 ⁻⁵	0.557	0.025
Threonine (6288)	0.021 (0.014)	0.011 (0.007)	0.003	0.381	0.017
Tridecanoate (12530)	2.4x10 ⁻⁴ (1.0x10 ⁻⁴)	3.8x10 ⁻⁴ (1.7x10 ⁻⁴)	9.5x10 ⁻⁶	0.562	0.010
Tyrosine (6057)	0.019 (0.008)	0.014 (0.006)	0.007	0.343	0.018
Valine (6287)	0.188 (0.115)	0.094 (0.061)	9.3x10 ⁻⁴	0.421	0.027

4.5. DISCUSSION

The altered metabolite markers listed in Table 4-2 are indicative of the major metabolic pathways affected by the marathon and are mainly associated with the macro-fuel substrate utilisation pathways (carbohydrates, lipids and AAs) and the regulation thereof (TCA cycle, oxidative phosphorylation and GM). These metabolite pathways, along with the intermittent dietary-associated metabolite markers, are comprehensively discussed below and schematically presented in Figure 4-2.

Various carbohydrate metabolites were significantly elevated following the marathon and can be ascribed to gluconeogenic influx (MacLaren & Morton, 2012) and reduced insulin secretion (Richter *et al.*, 1992), which is typically induced by an initial depletion of glucose and glycogen stores occurring approximately 90 min after the start of a marathon (exercise intensity-dependent) at a VO_{2max} >75% (Stellingwerff, 2012). The reduced insulin concentrations temporarily inactivate insulin-dependent glucose uptake systems (i.e. GLUT 4 transporters and glucokinase) (Salway, 2012), preventing glucose absorption into cells. This could ascribe the post-marathon elevations in serum glucose concentrations and various other associated metabolites, including glucarate (Żółtaszek *et al.*, 2008) and mannose (Hu *et al.*, 2016) as well as the elevated concentrations of gluconeogenesis-associated metabolites, i.e. myo-inositol (Eisenberg & Parthasarathy, 1987), erythritol (synthesised via erythrose-4-phosphate in pentose pathway) (Hootman *et al.*, 2017), glycerol and glycerate

(Wadman *et al.*, 1976; Salway, 2012). Furthermore, the elevated concentrations of pyruvate observed in the post-marathon samples were anticipated since it is an end-product of the glycolysis pathway which feeds into the TCA cycle for further ATP production (Salway, 2012). This is confirmed by the accumulation of various TCA cycle intermediates such as α -ketoglutarate, succinate, citrate, fumarate and malate (Qiang, 2015), which also indicate the accumulation of circulating NADH/FADH₂ molecules (Esterhuizen *et al.*, 2017) as a result of a saturated ETC activity. It is also important to mention that many of the aforementioned carbohydrate metabolites, along with elevated concentrations of mannose (Hu *et al.*, 2016), sorbose (Guzik & Stachowicz, 2016), mannitol (McNutt, 2000), tagatofuranose (Kroger *et al.*, 2006), and threonate (an ascorbate derivative) (Simpson & Ortwerth, 2000), are well-known constituents of fruits/fruit juices, vegetables/vegetable juices, peanuts, energy bars, energy drinks and various other ergogenic aids consumed by the athletes during the course of the marathon (Jeukendrup, 2011; Pfeiffer *et al.*, 2012).

The reduction in intracellular glucose due to the aforementioned cellular uptake inhibition is known to activate lipolysis of free and adipose tissue-bound TAG (MacLaren & Morton, 2012) as alternative fuel substrates. This is substantiated by the elevated concentrations of serum glycerol, monopalmitin and various free FAs (laurate, palmitate, palmitoleate, 11-eicosenoate, 11,14-eicosadienoate, myristoleate, α -linolenate, 5-dodecenoate, linoleate and oleate) (Lewis *et al.*, 2010; Waśkiewicz *et al.*, 2012; Kujala *et al.*, 2013; Peake *et al.*, 2014). Additionally, accumulated 3-hydroxy acids (β -hydroxyhexanoate) and 3-keto acids (β -hydroxy- α,β -didehydrosebacate) are indications of a saturated β -oxidation pathway, ascribed to the inhibition of the rate-limiting enzyme, β -hydroxyacyl dehydrogenase, which is pursued by β -ketoacyl-CoA thiolase. This saturated β -oxidation pathway results in the catabolism of the accumulated FAs via α -oxidation (Roe & Ding, s.a.), thus substantiating the elevated concentrations of α -hydroxyoctanoate (C₈; an α -oxidation intermediate) and the OCFA (tridecanoate [C₁₃], pentadecanoate [C₁₅], heptadecanoate [C₁₇] and 10-heptadecenoate [C_{17:1}]) detected in the post-marathon serum. It should however be mentioned that these OCFA may also arise from elevated dietary intake (Jenkins *et al.*, 2017) and/or autophagy of various cellular constituents during extensive energy-requiring states (Singh & Cuervo, 2011). Nevertheless, irrespective of their origins, these OCFA are ultimately catabolised to propionyl-CoA (Pfeuffer & Jaudszus, 2016), hence the elevated β -hydroxypropionate observed in the post-marathon serum. The elevated concentrations of β -hydroxybutyrate and acetoacetate are anticipated, as these are alternative fuel substrates for the brain (Cahill & Vech, 2003) and skeletal muscles (Holloszy & Coyle, 1984) in hypoglycemic states, and could also be an indication of an imbalanced redox state (Salway, 2012; Esterhuizen *et al.*, 2017). Furthermore, the post-marathon elevations of malonate typically indicate the accumulation of malonyl-CoA, which is a LCFA transport inhibitor (Salway, 2012) and could therefore be an additional reason for the increased cytosolic LCFAs (palmitate, palmitoleate, 11-eicosenoate, 11,14-eicosadienoate, myristoleate, α -linolenate, linoleate and oleate).

Most AAs are catabolised into TCA cycle substrates via propionyl-CoA, succinyl-CoA, pyruvate or acetyl-CoA, depending on the specific AA (Salway, 2012). Reduced concentrations of AAs (serine, glycine, alanine,

aspartate, phenylalanine, tyrosine, threonine and methionine) and altered AA-associated metabolite concentrations (dimethylglycine (Holm *et al.*, 2003), pyroglutamate (Kumar & Bachhawat, 2012), indole-3-acetate (Salway, 2012) and glutarate (Peake, 2016)) were detected in the post-marathon serum, which indicates AA catabolism during the marathon. Furthermore, elevated concentrations of α -hydroxybutyrate (an intermediate in the threonine/methionine pathway), further supports the aforementioned NADH:NAD⁺ imbalance, ketoacidosis, reduced insulin secretion and impaired glucose absorption (Gall *et al.*, 2010). Additionally, elevated *p*-hydroxyphenylacetate and *p*-hydroxyphenyllactate could be indicative of mild liver injury/damage (Liebich & Pickert, 1985; Ghoraba *et al.*, 2014), which concur with previous findings (Lippi *et al.*, 2011; Jastrzebski *et al.*, 2015).

In accordance with previous findings (Pechlivanis *et al.*, 2010), reduced serum concentrations of the BCAAs isoleucine (0.05 vs 0.02 $\mu\text{g/mL}$, $p=0.018$), valine and leucine were detected in the post-marathon samples, while their catabolism intermediates i.e. α -ketoisovalerate, β -hydroxyisobutyrate (valine metabolites) and α -ethylhydracrylate (allo-isoleucine metabolism) (Wendel *et al.*, 1989) were significantly elevated. Allo-isoleucine catabolism intermediates may serve as alternative substrates in the valine catabolism pathway (Ryan, 2015), however, since the valine catabolism intermediates remained elevated in the post-marathon serum it can be deduced that allo-isoleucine is instead catabolised to α -ethylhydracrylate (Korman *et al.*, 2005) via α -keto- β -methylvalerate (explaining its reduced concentrations post-marathon) with the subsequent production of FADH₂ (Korman *et al.*, 2005). The elevated concentrations of β -hydroxyisovalerate detected post-marathon are typically associated with ketone rich environments (as observed in the current investigation), resulting from increased leucine catabolism and the subsequent isovaleryl-CoA production (Mock *et al.*, 2011). This metabolite is also a well-known constituent of athlete supplementation (Brioche *et al.*, 2016) and could be elevated due to dietary ingestion during the marathon. Furthermore, reduced concentrations of these BCAAs, in particular leucine, results in mTORC 1 inhibition (Laplane & Sabatini, 2009), which in turn activates various catabolic processes such as autophagy (of organelle and plasma membrane constituents) to release additional embedded fuel substrates (Singh & Cuervo, 2011). mTORC 1 inhibition can also be induced by other factors including elevated 5'-AMP-activated protein kinase during energy deprivation, reduced oxygen levels, reduced essential AAs and inflammation (Laplane & Sabatini, 2009), all of which are associated with endurance races and evidently occur in the marathon athletes investigated in this study. Considering that autophagy contributes to elevated amounts of cellular debris, the aforementioned accumulation of FAs and especially the OCFAs, in the post-marathon serum may also be explained by the autophagosomal degradation of phospholipids, sphingolipids (Kishimoto *et al.*, 1973; Maes *et al.*, 1996) and phytosphingosines (Kondo *et al.*, 2014; Kitamura *et al.*, 2017) found in cell and organelle membranes. Additionally, the elevated concentrations of squalene and 5-pregnene-3 β ,20 α -diol in the post-marathon serum suggest cholesterol degradation (Charlton-Menys & Durrington, 2007; Salway, 2012), further supporting the activation of autophagy as the latter is a common constituent in cell membranes (Salway, 2012). Elevated 5-pregnene-3 β ,20 α -diol (also known as 20 α -dihydropregnenolone) post-marathon also indicates

steroid metabolism activation via cholesterol catabolism as it is produced by the reduction of pregnenolone via 20-hydroxysteroid dehydrogenase (Ebner *et al.*, 2006). Pregnenolone is a precursor for the production of aldosterone and cortisol, the latter of which stimulates lipolysis, gluconeogenesis and protein catabolism (Salway, 2012).

Furthermore, a significant decrease in α -aminomalonate concentrations was observed in the post-marathon serum samples. Although the precise origin of this metabolite is unknown, it has been associated with abnormal protein oxidation, macrophage accumulation, non-essential AA oxidation via ROS pulmonary arterial hypertension indication (Bujak *et al.*, 2016).

The microbiome plays a crucial role in many essential metabolic processes required during strenuous exercise, including the regulation of energy metabolism, oxidative stress and inflammatory response pathways (Mach & Fuster-Botella, 2017). To this end, various microbial-associated metabolites (i.e. tagatofuranose, talofuranose, ethyl- α -D-glucopyranoside, arabinol, indole-3-propionate and D-rhamnose) were significantly altered in the post-marathon samples. Most of these metabolites are synthesised from host carbohydrate intermediates, either via catabolism or fermentation processes (arabinol: sugar alcohol produced from arabinose/glucose (Kumdam *et al.*, 2014); and ethyl- α -D-glucopyranoside: an ethanolysis product from glucose (Hu *et al.*, 2013)). Indole-3-propionate is the deamination product of tryptophan, produced by gut microbes (e.g. *Clostridium sporogenes*) (Wikoff *et al.*, 2009) and further supports the notion of reduced AAs. The reduced concentrations of D-rhamnose, a component of most Gram-positive bacterial cell walls (Mistou *et al.*, 2016), contradict the above-mentioned microbial product elevations. Since the particular nature of this reduction following completion of the marathon is unclear, it warrants future investigation.

In addition to these endogenously and microbially produced metabolites, elevated concentrations of ibuprofen were also observed post-marathon. Ibuprofen is a well-known non-steroidal, anti-inflammatory drug (Nieman *et al.*, 2006) commonly used by athletes for preventing muscle damage, muscle soreness and inflammation and was most likely consumed by some athletes prior to/during the marathon (McAnulty *et al.*, 2007).

4.6. CONCLUSION

The results of this metabolomics investigation suggest that the body utilises various fuel substrate pathways to comply with the high energy demands required during the marathon, including catabolism of carbohydrates, lipids (β -oxidation and α -oxidation) and AAs, as well as activation of ketogenesis and autophagy via mTOR1 inhibition. Considering the results of the current investigation as well as previous literature, the possible cascade of events contributing to this metabolic “snapshot” could be summarised as follows: (1) A proposed initial reduction in carbohydrate catabolism and glucose uptake via the insulin-dependent transporters lead to glycolysis dysregulation, ketogenesis activation and increased serum glucose. (2) A metabolic shift towards FA utilisation (from either endogenous or dietary TAGs) is induced, which (3) overwhelms/saturates the β -oxidation pathway, resulting in the α -oxidation of FAs. (4) AAs (from either endogenous or dietary protein catabolism) are also used as alternative fuel substrates, resulting in (5) mTOR1 inhibition and autophagy as the body desperately tries to generate the necessary fuel substrates to comply with the energy demand. (6) Lastly, various metabolic processes are activated to reduce oxidative stress and regulate/correct the redox imbalance.

4.7. LIMITATIONS AND FUTURE PROSPECTS

Possible limitations of this study include human genotype/phenotype variation (an inevitable confounder) and the uncontrolled dietary intake of the athletes during the marathon. However, convincing athletes to deviate from their individualised supplementation protocols would be extremely difficult, if not impossible. Validation using a larger sample cohort could further substantiate the current findings. Nonetheless, these findings indicate the extensive metabolic changes induced by the marathon perturbation. Possible future prospects could be to investigate the effects of supplementing with AAs, pre- and probiotics, and β -hydroxyisovalerate as a means of improving aerobic exercise performance, reduce skeletal muscle and liver damage and enhance recovery.

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CHAPTER 5: UNAIDED METABOLIC RECOVERY OF ATHLETES

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5.1. ABSTRACT

Endurance athlete performance is greatly dependent on sufficient post-race system recovery, as endurance races have substantial physiological, immunological and metabolic effects on these athletes. To date, the effects of numerous recovery modalities have been investigated, however, very limited literature exists pertaining to metabolic recovery of athletes after endurance races without the utilisation of recovery modalities. As such, this investigation is aimed at identifying the metabolic recovery trend of athletes within 48 h after a marathon. Serum samples from 16 athletes collected 24 h before, immediately after, as well as 24 h and 48 h post-marathon were analysed using an untargeted GCxGC-TOFMS metabolomics approach. The metabolic profiles of these comparative time-points indicated a metabolic shift from the overall post-marathon perturbed state back to the pre-marathon metabolic state during the recovery period. Statistical analyses of the data identified 61 significantly altered metabolites including AAs, FAs, TCA cycle, carbohydrate and associated intermediates. These intermediates recovered to pre-marathon related concentrations within 24 h post-marathon, except for xylose which only recovered within 48 h. Furthermore, fluctuations in cholesterol and pyrimidine intermediates indicated the activation of alternative recovery mechanisms. Metabolic recovery of the athletes was attained within 48 h post-marathon, most likely due to reduced need for fuel substrate catabolism. This may result in the activation of glycogenesis, uridine-dependent nucleotide synthesis, protein synthesis, and the inactivation of cellular autophagy. These results may be beneficial in identifying more efficient, targeted recovery approaches to improve athletic performance.

5.2. INTRODUCTION

Endurance races (>5 km) have become increasingly popular over the last decade and primarily include half-marathons (21.1 km), marathons (42.2 km), and ultra-marathons (>42.2 km) (Lippi et al., 2012). Participation in these events requires extensive preparation including physical and mental conditioning as well as meticulously planned dietary strategies. According to Barnett (2006), optimal athletic performance is only achieved when these principles are accompanied by sufficient system recovery following any endurance activity. “Recovery” comprehensively refers to the process by which an altered biological system, such as the metabolome, reverts to its corresponding pre-perturbed state (Tomlin & Wenger, 2001). In endurance athletes, metabolic recovery is thought to proceed in a biphasic manner with the initial phase mainly consisting of rapid oxygen, ATP and phosphocreatine replenishment, whereas the second phase entails the slow restoration of innate metabolism adaptations (Tomlin & Wenger, 2001; Barnett, 2006). A popular research approach for identifying and mapping such metabolic adaptations quantitatively and qualitatively is collectively referred to as “metabolomics”. Considering that the metabolome provides a direct depiction of the physiological state of an organism at a specific point in time (Heaney et al., 2017), metabolomics is a formidable tool when investigating the effects of endurance races on the human body, as well as the recovery thereof.

Previous studies investigating the perturbed metabolism of endurance athletes mainly observed elevated concentrations of carbohydrates, FAs, and ketones, accompanied by reduced concentrations of AAs (Davison et al., 2018). This is proposed to result from the dramatic metabolic shift between numerous fuel catabolism systems, as well as alternative energy-producing mechanisms (α -oxidation and autophagy) activated during strenuous activity (Lewis et al., 2010; Waśkiewicz et al., 2012; Nieman et al., 2013; Daskalaki et al., 2014; Stander et al., 2018). Additionally, the extensive reduction in fuel substrates was associated with alterations in the urea cycle, purine, steroid and pyrimidine metabolism, ROS production pathways (Lewis et al., 2010; Kujala et al., 2013; Daskalaki et al., 2014; Turer et al., 2014; Clifford et al., 2017b; Davison et al., 2018; Stander et al., 2018), as well as the GM (Daskalaki et al., 2014; Mach & Fuster-Botella, 2017).

Given the nature and extent of the physiological, immunological and metabolic effects of endurance exercise on the human body, a substantial amount of research has been directed towards investigating various therapeutic recovery modalities including cryotherapy, massage, heat therapy, stretching, compression, and NSAID (Barnett, 2006; Howatson & Van Someren, 2008), as well as the ingestion of functional foods, such as cherries, pomegranates, and green leafy vegetables (Harty et al., 2019). However, limited literature is available regarding the overall metabolic recovery of the perturbed metabolism after an endurance race, without the ingestion and/or utilisation of recovery modalities. From these studies, it is evident that during the immediate hours after recovery, myocellular glycogen restoration (Bielinski *et al.*, 1985; Egan & Zierath, 2013), as well as phosphocreatine store replenishment and blood lactate removal takes preference (Tomlin & Wenger, 2001; Barnett, 2006; Bishop *et al.*, 2008), while lipids are temporarily further oxidised as an energy source (Bielinski *et al.*, 1985; Egan & Zierath, 2013). Considering this, it is suggested that glycogen restoration is attained

within 24 h–48 h after endurance exercise (Egan & Zierath, 2013), whereas AAs, FAs, FA-carnitine conjugates along with TCA cycle and pyrimidine/purine pathway intermediates remain perturbed for at least 14 h after ER (Lewis *et al.*, 2010; Nieman *et al.*, 2013; Daskalaki *et al.*, 2014; Davison *et al.*, 2018).

Considering the current lack of literature regarding the metabolic recovery of marathon athletes over an extended period (48 h), an untargeted GCxGC-TOFMS serum metabolomics approach was used to investigate the unaided (without the ingestion and/or utilisation of recovery modalities) metabolic recovery (to baseline-related concentrations) of 16 marathon athletes by 48 h post-marathon. These results might improve the current knowledge pertaining to the consequence of endurance activity and the time-course of recovery in the days following the event, as well as lead to the identification of new or additional recovery pathways and/or more targeted, perhaps even personalised, recovery approaches.

5.3. METHODS AND MATERIALS

5.3.1. Participants and clinical samples

Considering that this section is aimed at elucidating the unaided metabolic recovery trend of athletes within 48 h after completing a marathon (Figure A-1), only serum samples of the 16 placebo-ingesting athletes (10 males and 6 females) were included in this part of the investigation (Figure 3-1). Chapter 3 contains a comprehensive overview of the participant selection and/or eligibility criteria (Section 3.2.2), marathon layout (Section 3.2.3), as well as sample collection and pre-processing procedures (Section 3.2.4). Furthermore, Table 3-1 (Chapter 3) provides a detailed summary of the characteristics of the placebo cohort, while Table A-2 (Annexure A) illustrates the age, sex, and overall performance distribution of the athletes. Besides these confounding factors, all athletes were instructed to abstain from using any recovery modalities/therapies such as cryotherapy, pressure garments, foam rolling, active recovery (moderate exercise bouts), NSAID, vitamins, etc. during the recovery period of this investigation.

5.3.2. Sample analysis

As previously described (Chapter 3), all samples along with pooled QC samples were subjected to a total metabolome extraction, before being derivatised (Section 3.2.6) and analysed by the Pegasus 4D GCxGC-TOFMS analytical apparatus (Section 3.2.7). After analytical analysis, the data generated was processed using the ChromaTOF software (Section 3.2.7).

5.3.3. Data clean-up and statistical analyses

After the normalisation of compounds relative to the internal standard, and various data-clean-up (Section 3.3.3) the data was subjected to a multi-statistical approach consisting of two main objectives i.e. (1) obtaining a global pattern of the metabolic profile of athletes over time, and to some extent validate the design against

the logical result, as well as (2) determining the specific metabolic adaptations between time points (Figure 5-1) to distinguish between sources of variation due to early (24 h post- vs post-marathon) and late (48 h post vs post-marathon) stages recovery; from full recovery (48 h post- vs pre-marathon). As such, statistical analyses consisted of both paired univariate and multivariate approaches, using MATLAB (2012) with a PLS toolbox (2016). In terms of the multivariate analyses, an ML-PCA were used to inspect specific time-point differences in the multivariate space, whereas an analysis of variance (ANOVA) simultaneous component analysis (ASCA) was employed to summarise the overall metabolic perturbation observed over the entire timeframe of the study (Smilde *et al.*, 2005). Both these methods take the dependence between observations into account. Furthermore, univariate data analyses included a paired *t*-test, corrected for multiple testing by controlling the FDR (limiting to 5%) using the Benjamini-Hochberg procedure (Benjamini & Hochberg, 1995), and a dependent effect size test to respectively assess the statistical and practical significance of each metabolite (Field, 2011). Considering the inevitable confounder of inter-individual variation, a repeated measures statistical design was employed to control for the latter as each athlete's metabolic variation is evaluated relative to him/herself. That said, statistical approaches for paired data may not correct for all confounders, therefore this design aimed to ensure equal representation across different confounders, as indicated in Table A-1 (Annexure A). Although more complex models were considered for variable selection (i.e. repeated measures ANOVA), these models would not be applicable due to the small sample size and were therefore excluded.

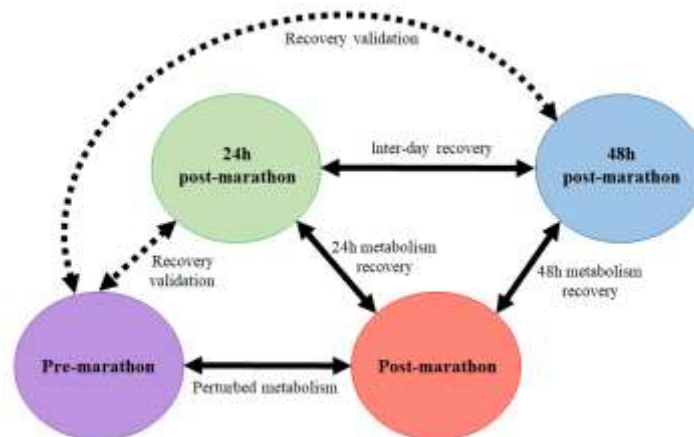


Figure 5-1: Schematic representation of the sample group comparisons performed during this investigation.

5.4. RESULTS

5.4.1. Multivariate statistical outputs

In order to visualise the overall metabolic shift over the entire timeframe of the study, an ASCA model was constructed with the resulting scores graphically displayed and grouped according to the time component of the current study design (Figure 5-2). All scripts used here are readily available in the mentioned toolboxes

provided by Eigenvector Research Inc., with the exception of the ASCA scripts, which can be downloaded from <http://www.bdagroup.nl/content/Downloads/software/software.php>. Based on Figure 5-2, it is evident that although a marathon has a dramatic impact on the human serum metabolome, these changes recover within 48 h, as indicated by the confined positioning of the 24 h and 48 h post-marathon ellipsoids relative to that of the pre-marathon. Furthermore, in an attempt to inspect specific adaptations between time-points, individual ML-PCA plots for the specified group comparisons (Figure 5-1) are presented in Annexure A (Figure A-2 a–f). The following can be deduced from these figures (Figure A-2 a–f): (1) As expected, the marathon perturbation (Figure A-2 a) induced significant metabolic alterations, which is supported by Stander *et al.* (2018); (2) Clear separation (no overlap) of the recovery time points (24 h and 48 h post-marathon) and post-marathon ellipsoids (Figure A-2 d and e) indicate a significant deviation from the post-marathon perturbed metabolic state; (3) The magnitude of metabolome variation presented 24 h and 48 h post-marathon seems rather minor as these ellipsoids appear relatively grouped (ellipsoid overlap); (4) The confined positioning of the 24 h and 48 h post-marathon ellipsoids relative to the pre-marathon indicate the recovery of the metabolome to a baseline-related state (overlapping ellipsoids).

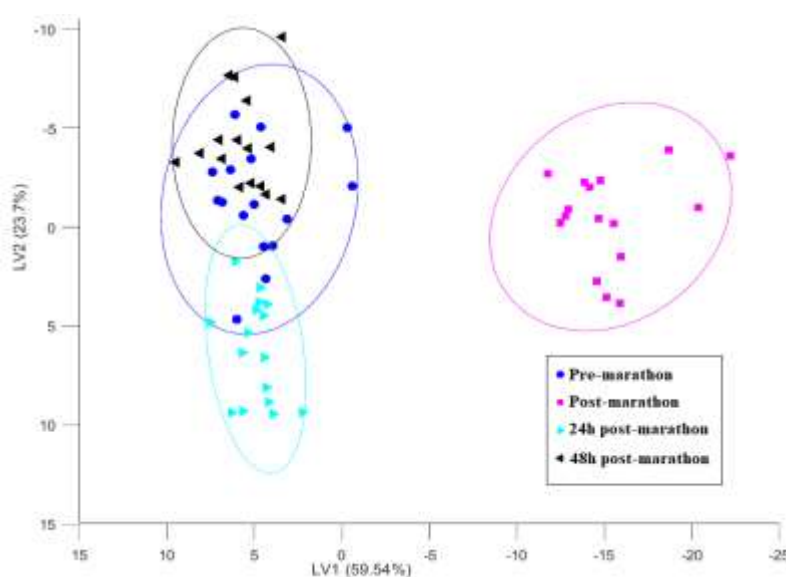


Figure 5-2: The ASCA plot indicating the natural, time-dependent differentiation of the comparative groups: Pre-marathon (denoted by blue/circle), post-marathon (denoted by pink/square), 24 h post-marathon (denoted by turquoise/right-tilted triangle) and 48 h post-marathon (denoted with black/left-tilted triangle). The variance accounted for by each latent variable (LV) is indicated in parenthesis on the respective axes. The ellipsoids represent 95% confidence intervals of each time points centroid.

Furthermore, in order to visualise metabolite stratification among the comparative groups, clustering dendrograms were constructed and are included in the supplementary material (Figure A-3). From these figures, it is clear that stratification associations did not correlate with the comparative groups.

However, considering that controlling for model overfitting, the likelihood of increased FDR (high dimensionality), and the relatively small sample set, multivariate models could not be validated and was not used for variable selection, but rather for global metabolic shift visualisation.

5.4.2. Univariate statistical outputs

Considering the aforementioned, variable selection was based on univariate comparisons as a means of determining the specific metabolic adaptations apparent during the course of this investigation while controlling the FDRs and avoiding overfitting. Although repeated measures ANOVA models usage may have been appropriate, paired t-tests were preferred since the dramatic effect of the marathon would result in a large number of significant metabolites requiring pairwise comparisons as post-hoc tests in any event. Based on this, only metabolites with a Benjamini-Hochberg p -value ≤ 0.05 and an effect size d -value ≥ 0.5 (moderate effect) in each group comparison (Figure 5-1), were deemed to be statistically significant. This approach yielded a list of 74 metabolite markers, of which 61 were positively annotated (the remaining 13 metabolites were classified as “Unknowns/Analytes”) and are listed in Table 5-1. A more comprehensive table including average concentrations over the comparative time points has been included in Annexure A (Table A-2).

Table 5-1: Ratio of concentration fluctuations in the serum metabolite markers ($n=61$) best describing the detected variation and metabolic recovery of the athletes within 48 h post-marathon.

Compound name (PubChem ID)	Post/pre-marathon ratio	24 h post-/post-marathon ratio	48 h post-/post-marathon ratio	48 h post-/24 h post-marathon ratio	24 h post-/pre-marathon ratio	48 h post-marathon/pre-marathon ratio
Carbohydrate and associated intermediates						
Arabitol (439255)	1.36*	0.75*	0.65*	0.87	1.01	0.88
Erythritol (222285)	1.77*	0.61*	0.39*	0.63	1.08	0.68
Glucose (5793)	8.91*	0.17*	0.09*	0.52	1.48	0.77
Mannitol (6251)	1.34	0.78	0.58*	0.75	1.05	0.78
Mannose (18950)	15.72*	0.11*	0.05*	0.49	1.66	0.82
Myo-inositol (440194)	1.10	0.76*	0.63*	0.82	0.84	0.69
Psicose (441036)	1.15	0.45*	0.70	1.56	0.52	0.80
Rhamnose (5460029)	0.62*	1.51	1.86*	1.23	0.94	1.16
Ribose (10975657)	1.16	0.58*	0.89	1.53	0.67	1.03
Sorbose (439192)	1.46	0.69	0.64*	0.94	1.01	0.94
Tagatose (439312)	1.87*	0.56*	0.53*	0.95	1.04	0.99
Talose (441035)	1.69*	0.57*	0.52*	0.91	0.96	0.87
Threonate (151152)	1.39*	0.80*	0.78*	0.98	1.11	1.09
Uridine (6029)	1.06	0.61*	1.02	1.66*	0.65	1.08
Xylose (135191)	0.90	0.52*	0.76	1.45	0.47*	0.69
Lipid and associated intermediates						
α -Hydroxyoctanoate (94180)	1.49*	0.63*	0.67	1.06	0.94	0.99
α -Linolenate (5280934)	1.26*	0.69*	0.74*	1.07	0.87	0.93

β -Hydroxyhexanoate (11829482)	3.00*	0.38*	0.31*	0.82	1.13	0.92
5-Dodecenoate (5312377)	35.22*	0.02*	0.06*	3.73	0.59	2.19
10-Heptadecenoate (5312434)	5.87*	0.12*	0.15*	1.22	0.73	0.89
11,14-Eicosadienoate (5282805)	1.94*	0.54*	0.45*	0.83	1.05	0.87
11-Eicosenoate (5282768)	3.48*	0.31*	0.37*	1.20	1.06	1.28
Docosahexaenoate (445580)	1.17	0.69*	0.80	1.17	0.80	0.94
Eicosapentaenoate (446284)	1.30	0.69*	0.74*	1.07	0.90	0.96
Glycerol (753)	5.84*	0.17*	0.19*	1.11	0.99	1.10
Glycerol monopalmitate (3084463)	1.87*	0.44*	0.55*	1.24	0.82	1.02
Heptadecanoate (10465)	1.82*	0.52*	0.52*	1.01	0.94	0.95
Laurate (3893)	2.07*	0.57*	0.46*	0.81	1.17	0.95
Methyl oleate (9922235)	1.68*	0.48*	0.72	1.51	0.81	1.22
Myristoleate (5281119)	12.33*	0.06*	0.08*	1.32	0.70	0.93
Oleate (445639)	5.04*	0.21*	0.22*	1.07	1.06	1.13
Palmitate (985)	1.67*	0.55*	0.57*	1.04	0.93	0.96
Palmitoleate (445638)	6.26*	0.19*	0.22	1.17	1.20	1.40
Pentadecanoate (13849)	2.21*	0.51*	0.43*	0.85	1.13	0.96

Ketones and TCA cycle intermediates

α -Ketoglutarate (51)	1.57*	0.58*	0.64*	1.11	0.91	1.01
β -Hydroxybutyrate (441)	12.98*	0.06*	0.06*	1.12	0.72	0.81
Acetoacetate (96)	2.89	0.37*	0.19*	0.51	1.07	0.54
Citrate (311)	1.46	0.74	0.58*	0.78	1.08	0.85
Fumarate (444972)	1.42	0.75	0.68*	0.91	1.06	0.96
Malate (525)	1.62*	0.57*	0.52*	0.90	0.93	0.84
Malonate (867)	1.37	0.72	0.62*	0.86	0.99	0.85

AA and cholesterol-associated intermediates

α -Ethylhydracrylate (188979)	2.24*	0.48*	0.47*	0.97	1.08	1.05
α -Hydroxybutyrate (11266)	2.53*	0.52*	0.49*	0.93	1.31	1.23
β -Hydroxyisobutyrate (87)	15.39*	0.04*	0.06*	1.29	0.67	0.87
β -Hydroxyisovalerate (69362)	1.15	0.89	0.75*	0.84	1.02	0.86
5-Pregnen-3 β ,20 α -diol (312224064)	1.93*	0.38*	0.36*	0.96	0.73	0.70
Alanine (5950)	0.64	2.34*	1.86*	0.79	1.51	1.19
Aminomalonate (100714)	0.48	2.10*	2.06*	0.98	1.01	0.99
Aspartate (5960)	0.60	2.03*	1.36	0.67	1.22	0.82
Glutamate (33032)	0.79	1.51*	1.39	0.92	1.19	1.10
Glutarate (743)	1.73	1.12	0.52*	0.46	1.94	0.89
Glycine (750)	0.59	1.79*	1.45	0.81	1.06	0.86
Hydroxyproline (5810)	0.44	2.03	2.68*	1.32	0.89	1.18
Methionine (6137)	0.54	2.17*	1.88*	0.87	1.18	1.02

Phenylalanine (6140)	0.64	1.93*	1.47*	0.76	1.24	0.95
p-Hydroxyphenyl-acetate (127)	2.00	0.51*	0.45*	0.88	1.03	0.90
p-Hydroxyphenyl-lactate (9378)	1.53	0.66*	0.58*	0.88	1.01	0.89
Pyroglutamate (7405)	0.71*	1.35*	1.41*	1.04	0.95	1.00
Squalene (638072)	3.39*	0.31*	0.34*	1.07	1.06	1.14
Tyrosine (6057)	0.64	1.78*	1.57*	0.88	1.14	1.01
Valine (6287)	0.53	1.83	2.21*	1.21	0.97	1.18

Footnotes: Significant *p*- and *d*-values (*).

5.5. DISCUSSION

Table 5-1 depicts the recovery trend of the metabolites not only initially affected by the marathon perturbation, but also the concentration fluctuations of additional metabolites pertinent to the 24 h and 48 h recovery period. In order to ascertain the metabolic recovery mechanisms following a marathon, it is imperative to first consider the immediate effects of a marathon on the serum metabolome of athletes, which was characterised and previously described by Stander *et al.* (2018). Briefly, alterations were mainly observed in metabolic pathways associated with energy production, such as elevated concentrations of various carbohydrates, TCA cycle intermediates, FAs and ketone bodies, and reduced concentrations of various AAs and associated metabolites. The perturbed metabolic state may also be linked to imbalanced redox potential, the saturation of traditional FA catabolic pathways, possible insulin secretion hampering, and the activation of cellular autophagy to provide additional fuel substrates.

A detailed description of the recovery (to pre-marathon related concentrations) of the identified metabolites are discussed in a grouped-manner based on metabolic alterations of the recovery time-points (24 h and 48 h post-marathon) relative to the perturbed metabolic state (post-marathon; immediately post-marathon) and is schematically summarised in Figure 5-3.

5.5.1. Carbohydrate metabolism

Carbohydrates, glucose, in particular, are considered to be the primary fuel substrates used by the body during physical activity. Stander *et al.* (2018) and Lewis *et al.* (2010) reported severely elevated serum concentrations of various carbohydrates immediately after a marathon (post-marathon). In this metabolomics investigation, it is clear that the majority of the serum carbohydrates (glucose and mannose) and associated metabolites (uridine, ribose, glycerol, and myo-inositol) returned to baseline (pre-marathon) concentrations within 24 h post-marathon (Table 5-1), with the exception of xylose (also dietary-related). This can be ascribed to the restoration of the glycogen stores, which were perceived as depleted in the initial stages of the marathon (Stander *et al.*, 2018).

Moreover, substantial reductions in uridine concentrations were observed within 24 h post-marathon (Table 5-1). Uridine (a nucleoside formed via the conjugation product of ribose and uracil) is a key component in RNA and subsequently protein synthesis (Yamamoto *et al.*, 2011). This, in conjunction with the reduced ribose, indicated possible increased nucleotide production via the activation of pyrimidine salvation metabolism. The pyrimidine salvation metabolism refers to the adaptive mechanism by which nucleotides can be synthesised using pre-formed nucleosides and nucleobases, usually following a nucleotide degradation period (Yamamoto *et al.*, 2011; Davison *et al.*, 2018), such as during the extensive protein catabolism induced by a marathon (Lewis *et al.*, 2010; Nieman *et al.*, 2013; Stander *et al.*, 2018). These recycled nucleotides can also interact with lipids to form pyrimidine nucleotide–lipid conjugates, a key component in cell membrane structures (Yamamoto *et al.*, 2011). Considering this, the slight reduction in uridine concentrations observed 24 h after the completion of the marathon, may also be associated with the restoration of damaged cellular membranes, which occurs as a result of strenuous exercise (Standar *et al.*, 2018). This is further substantiated by the reduction/recovery of myo-inositol concentrations within 24 h post-marathon, as it is a well-known constituent of glycerophospholipids (Christie & Han, 2012).

Additionally, the *de novo* pyrimidine synthesis pathway is dependent on various elements of the pentose phosphate pathway (Yamamoto *et al.*, 2011), particularly ribose-5-phosphate and phosphoribosyl pyrophosphate (Salway, 2012). Since these are interlinking substrates for the production of erythrose-4-phosphate (Salway, 2012), the recovery of erythritol (produced from the latter) (Hootman *et al.*, 2017) not only suggests the activation of glycogenesis via the utilisation of pentose phosphate pathway intermediates, but further supports the activation of the abovementioned nucleotide repair mechanisms. Furthermore, since the majority of the erythritol is absorbed rather than metabolised (Boesten *et al.*, 2013), as well as the fact that it is a well-known alternative sugar added to low-calorie foods (Chattopadhyay *et al.*, 2014) frequently ingested by athletes, the alterations of this metabolite 24 h and 48 h post-marathon may also be due to reduced ingestion of such foods by the athletes during the recovery period. Similarly, several of the metabolites in Table 5-1, including mannitol, psicose, sorbose (Chattopadhyay *et al.*, 2014; Yamada *et al.*, 2014), tagatose (Guerrero-Wyss *et al.*, 2018), and xylose (Salway, 2012), are natural constituents of fruit and vegetables, Food and Drug Administration approved alternative bulk sweeteners, and/or the products of metabolised sweeteners such as threonate (Thomas & Hughes, 1983), and arabitol via arabinose (Kumdam *et al.*, 2014). Considering that endurance races reportedly alter GM composition, as well as increase intestinal permeability resulting in GM metabolite migration (Rankin *et al.*, 2017), reductions in several of the detected metabolite markers (Table 5-1) (Kumdam *et al.*, 2014; Terami *et al.*, 2015) could also be ascribed to the restoration of GM homeostasis and a reduction in host substrate availability/dietary ingestion.

5.5.2. Lipid metabolism

The aforementioned depletion of intracellular glucose and glycogen stores, and the subsequent hampering of insulin secretion, may result in extensive lipolysis of TAG in adipose tissue, skeletal muscle and the liver

during strenuous activity (MacLaren & Morton, 2012; Davison *et al.*, 2018; Stander *et al.*, 2018). Recovery of these lipolysis intermediates (α -linolenate, 11,14-eicosadienoate, 11-eicosenoate, 5-dodecenoate, docosahexaenoate, eicosapentaenoate, laurate, myristoleate, oleate, palmitate, palmitoleate, glycerol, and glycerol monopalmitate) was observed within 24 h post-marathon (Table 5-1) and coincided with similar findings from previous literature (Lewis *et al.*, 2010; Nieman *et al.*, 2013; Davison *et al.*, 2018). The normalisation of these is a clear attestation of the down-regulation of lipolysis (Nieman *et al.*, 2013) mainly due to the aforementioned restoration of the glycaemic flux, concurrent with reduced ATP requirements during passive recovery (Baker *et al.*, 2010; MacLaren & Morton, 2012). The initial rapid reduction in serum FAs within 24 h post-marathon followed by a slight increase after 48 h post-marathon (Table 5-1) suggests a temporary upregulation of FA oxidation, succeeded by a short period of FA synthesis/re-esterification and/or a reduction in FA oxidation (Kimber *et al.*, 2003; Nieman *et al.*, 2013; Daskalaki *et al.*, 2015). According to Egan and Zierath (2013), myocellular glycogen replenishment is preferentially activated during the first 24 h–48 h post-exercise, subsequently causing a temporary increase in fat oxidation during this time, thus further supporting the reduction of FAs observed in this study. Withal, normalised concentrations of β -hydroxyhexanoate (a β -oxidation intermediate) within the 24 h post-marathon recovery period indicates the restoration of the previously mentioned β -oxidation and redox imbalances (Vidal *et al.*, 2017; Stander *et al.*, 2018), subsequently eliminating the need for alternative FA catabolism pathways, such as α -oxidation, which was previously proposed to be up-regulated during strenuous exercise (Standar *et al.*, 2018). This hypothesis is further supported by a reduction in α -hydroxyoctanoate (an α -oxidation intermediate) and the OCFAs (tridecanoate, pentadecanoate, heptadecanoate, and 10-heptadecenoate). However, it should be noted that reductions in OCFAs, as well as some of the aforementioned even-chain fatty acids, may also be ascribed to alleviated autophagy (Singh & Cuervo, 2011), reduced dietary ingestion of these, and an altered microbial activity (Jenkins *et al.*, 2017).

5.5.3. Ketone body production and the TCA cycle

The apparent reduced need for ATP post-marathon (Baker *et al.*, 2010) and the subsequently reduced fuel substrate catabolism during passive recovery is anticipated to cause a reduction in both acetyl-CoA and malonyl-CoA synthesis (MacLaren & Morton, 2012). The recovery of the malonate concentrations within 24 h post-marathon not only substantiates the latter as it coincides with the previously mentioned transient increase in LCFA oxidation but could also be an indication of said elevated FA synthesis. Furthermore, the restored concentrations of TCA cycle intermediates (α -ketoglutarate, fumarate, citrate, and malate) and ketones (acetoacetate and β -hydroxybutyrate) to that of pre-marathon concentrations within 24 h after the marathon (Table 5-1), support the aforementioned glycaemic flux recovery. This concurs with Nieman *et al.* (2013), who reported that these pathways remain perturbed for approximately 14 h post-exercise. Additionally, reduced concentrations of ketones, particularly β -hydroxybutyrate, support the restoration of the previously mentioned redox imbalance (Vidal *et al.*, 2017; Stander *et al.*, 2018) and suggest an enhanced metabolic

clearance rate (Evans *et al.*, 2017) in athletes within 24 h post-marathon. β -Hydroxybutyrate is also a signalling molecule serving as a regulator of gene expression and adaptive responses via the inhibition of class I histone deacetylases, rendering this metabolite invaluable to the recovery process (Evans *et al.*, 2017).

5.5.4. AA metabolism

In accordance with carbohydrates and lipids profile, various AAs (alanine, aspartate, glycine, glutamate, methionine, phenylalanine, and tyrosine) and associated metabolites (glutarate, *p*-hydroxyphenylacetate, *p*-hydroxyphenyllactate, pyroglutamate, and hydroxyproline) recovered to baseline concentrations within 24 h after the completion of the marathon (Table 5-1), indicating a reduced need for AA catabolism during this recovery phase. However, the slight reductions in these AAs that occur between 24 h and 48 h of recovery could be an indication of protein synthesis, as substantiated by the aforementioned uridine/ribose profile (Table 5-1). Contrarily, slight elevations in recovery time-point hydroxyproline may be indicative of delayed post-marathon collagen catabolism, thus supporting previous literature (Tofas *et al.*, 2008). Furthermore, the reduced concentrations of α -hydroxybutyrate (a threonine/methionine metabolism intermediate) within 24 h post-marathon compared to the elevated post-marathon (post-marathon) profile correlates with reduced AA catabolism and further suggests recovery of previously reported transiently hampered insulin secretion (Stander *et al.*, 2018), glucose absorption, and imbalance redox potential (Gall *et al.*, 2010).

In addition to the proteinogenic AAs, elevated concentrations of the BCAA valine, in conjunction with reduced BCAA-associated catabolism intermediates (Table 5-1) such as β -hydroxyisobutyrate (valine metabolism), α -ethylhydracrylate (allo-isoleucine metabolism), and β -hydroxyisovalerate (leucine metabolism), are indicative of a reduction in BCAA catabolism. BCAAs are reportedly one of the major factors regulating mTORC 1, a multi-protein complex responsible for the activation of numerous biological processes imperative to metabolic recovery, including cell proliferation, gene expression for lipid and protein synthesis, as well as the inhibition of cellular autophagy (Laplante & Sabatini, 2009). The proposed activation of these recovery mechanisms is further substantiated by the aforementioned uridine/ribose profile (Table 5-1), as well as the reduction in various cell membrane-associated intermediates, such as FAs (particularly OCFAs), myo-inositol and cholesterol intermediates (squalene and 5-pregnen-3 β ,20 α -diol) observed within 24 h post-marathon. Reduced concentrations of cholesterol intermediates within 24 h after the marathon in comparison to the post-marathon (post-marathon) concentrations also suggest inactivation of steroidogenesis (Ebner *et al.*, 2006), which was previously proposed to be induced as a result of endurance activity (Daskalaki *et al.*, 2015; Stander *et al.*, 2018).

Aminomalonate was also observed to recover to pre-marathon concentrations within 24 h post-marathon. Although this metabolite has been associated with oxidative damage to protein/AA residues (Bujak *et al.*, 2016), it has also been isolated from *Escherichia coli* (Van Buskirk *et al.*, 1984). As such, its precise mechanism of action pertaining to the metabolic recovery process remains to be elucidated.

5.6. CONCLUSION

The results of this investigation suggest that the serum metabolome of athletes largely recovers to baseline (pre-marathon) status within 24 h, with total recovery evident within 48 h post-marathon without the intervention of any recovery aids and other habitual practices. This can be ascribed to a reduction in ATP requirements during passive recovery, which in turn allows for the activation of anabolic recovery mechanisms or pathways. The possible cascade of events resulting in total metabolome recovery within 24/48 h post-marathon are: (1) reduced energy requirements promoting the restoration of the imbalanced redox potential and intracellular glycaemic flux, thus stimulating glycogenesis. (2) In turn, this further reduces the need for alternative fuel substrate catabolism (of lipids and AAs) and ketogenesis, (3) activating additional cellular repair mechanisms such as the uridine-dependent nucleotide salvage pathway, mTOR1 stimulation (subsequently inactivating cellular autophagy), as well as possible protein and lipid synthesis/FA re-esterification. Moreover, GM-associated markers emphasize the close metabolic interaction between man and microbe.

5.7. LIMITATIONS AND FUTURE PROSPECTS

Possible limitations of this investigation may include the inevitable confounder of human genotype/phenotype (sex, age and ethnicity) variation, as well as individual athlete dietary variation. Although the athletes' dietary intake was recorded throughout the investigation, adding dietary restrictions to the already refrained use of recovery aids would be considered too much of an interference to the personalised regimens of these athletes. However, in an attempt to circumvent the variability in participant demographics (age, sex, fitness, running experience, etc.), the study design included repeated measures of the same individuals and the use of statistical methods that consider the dependence between observations (therefore each participant becomes their own control) (Field, 2011; Howitt & Cramer, 2011). Nevertheless, such individual variation allows for a higher level of robustness in the results, which provides a better representation of the true nature of the activated recovery mechanisms as the body attempts to reach homeostasis.

Although the current investigation provided a holistic (untargeted) representation of the metabolome recovery after a marathon, future studies may consider applying more targeted approaches based on these results to possibly identify more personalised/efficient recovery modalities. Additionally, since this investigation was performed on a relatively small cohort, future investigations consisting of larger cohorts and/or serum samples obtained at additional time points (e.g. 12 h, 36 h, and 72 h post-marathon) are required to validate these findings, ultimately allowing for better metabolic mapping of the in-between stages via the identification of additional metabolite markers pertinent to the metabolic recovery trend of endurance athletes. Considering that metabolic differences correlating with gender heterogeneity could not be positively annotated due to limited cohort size in terms of this, future studies may consider including larger gender-specific cohorts. Lastly, since GM metabolism seems to be an integral part of athletic performance, more targeted analysis of

the associated metabolites or using an alternative biological medium (such as faecal matter, urine, etc.) could provide a more perspicuous understanding of the importance of the GM in terms of metabolome recovery.

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CHAPTER 6: BEETROOT JUICE AIDED METABOLIC RECOVERY OF ATHLETES

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6.1. ABSTRACT

Red beetroot (*Beta vulgaris L.*) is a dynamic functional food that reportedly exhibits potent anti-inflammatory, antioxidant, vasodilation, and cellular regulatory properties. As such, this vegetable has gained a fair amount of scientific attention as a possible cost-effective supplement to enhance performance and expedite recovery after physical exercise. To date, however, no study has investigated the effects of incremental beetroot juice ingestion on the metabolic recovery of athletes after an endurance race. Considering this, as well as the beneficial glucose and insulin regulatory roles of beetroot, this study investigated the effects of beetroot juice supplementation on the metabolic recovery trend of athletes within 48 h after completing a marathon. By employing an untargeted GCxGC-TOFMS approach, serum samples (collected pre-, immediately post-, 24 h post-, and 48 h post-marathon) of 31 marathon athletes who ingested a series of either beetroot juice or placebo supplements post-marathon, were analysed and statistically compared. From the results, it is evident that the metabolic profiles of the beetroot-ingesting cohort recovered to a pre-marathon-related state within 48 h post-marathon, mimicking the metabolic recovery trend observed in the placebo cohort. Nonetheless, since random inter-individual variation was observed immediately post-marathon, only metabolites with large practical significance within 24 h and 48 h post-marathon were considered representative of the effects of beetroot juice on metabolic recovery. These mainly included carbohydrates and OCFA. Although the majority of these were attributed to beetroot content, a great deal of microbial dependence was apparent in these fluctuations. Regardless, it was concluded, that in the current investigation, beetroot juice did not provide any significant advantages towards expediting metabolic recovery within 48 h post-marathon, when compared to the placebo cohort (unaided recovery).

6.2. INTRODUCTION

In addition to the well-established physiological (Knechtle & Nikolaidis, 2018) and immunological (Nieman & Pence, 2019) health effects of ER races (>5 km), this sport induces a wide variety of metabolic adaptations (Daskalaki *et al.*, 2014; Stander *et al.*, 2020b). These adaptations are primarily associated with fuel substrate catabolism and typically include elevated glycolysis, lipolysis, AA oxidation, ketogenesis, and TCA cycle intermediates (Lewis *et al.*, 2010; Li *et al.*, 2018; Stander *et al.*, 2018; Górecka *et al.*, 2019; Schader *et al.*, 2020). Additionally, previously reported elevations in dicarboxylic acids, α -hydroxy acids and OCFAs coupled with reduced phospholipids, suggest the activation of alternative energy-producing pathways such as ω and/or α -oxidation of fatty acids, as well as autophagy of cellular membranes (Nieman *et al.*, 2017; Stander *et al.*, 2018). These adaptations, along with fluctuations in purine and pyrimidine, urea cycle, and reactive oxygen intermediates (Lewis *et al.*, 2010; Howe *et al.*, 2018; Li *et al.*, 2018; Stander *et al.*, 2018; Schader *et al.*, 2020) not only further attest to the extensive energy-taxing nature of principal component, but also emphasize the metabolic flexibility of athletes to use multiple fuel substrates during endurance races, to supply the necessary energy to complete the event. Based on previous literature, these metabolic adaptations recover to pre-exercise-related levels within 3–72 h after cessation of endurance exercise (Nieman *et al.*, 2013; Daskalaki *et al.*, 2015; Davison *et al.*, 2018; Stander *et al.*, 2020a), depending on the intensity, duration, and distance of the running event. Post-exercise metabolic restoration is mainly attributed to a reduction in energy requirements, which leads to the inactivation of catabolic pathways required for energy production, and the activation of anabolic pathways required for recovery, including the nucleotide salvage pathways, glycogenesis, lipogenesis and protein synthesis (Nieman *et al.*, 2013; Davison *et al.*, 2018; Stander *et al.*, 2020a).

Besides the potentially detrimental, performance diminishing effects induced, endurance races are still considered a popular leisure activity, globally. As such, a growing body of research is aimed at identifying more cost-effective strategies that may expedite the recovery process, and subsequently enhancing athlete performance. One of these strategies includes pre- and/or post-exercise ingestion of supplements that either consists of or contains functional foods. Functional foods are primarily defined as any natural and/or fortified foods, rich in bioactive compounds that possess an added advantage to human health besides its nutritional value (Kareb & Aider, 2019; Tarabella *et al.*, 2019). Bioactive phytonutrients (i.e. polyphenols, carotenoids, curcuminoids, anthocyanins, vitamins, etc.) found in fruits and vegetables, reportedly enhance and maintain cardiovascular health, reduce cholesterol, while also preventing DNA damage, diabetes and certain cancers (Hadipour *et al.*, 2020; Nahar *et al.*, 2020; Thakur *et al.*, 2020). Besides these beneficial health attributes, phytonutrient-rich fruits and vegetables are also well-known for their radical scavenging, antioxidant, anti-inflammatory and anti-viral/microbial properties (Forni *et al.*, 2019) that may aid in performance-enhancing and/or physiological recovery of athletes during and/or after exercise. To date, some of the foods that have been investigated for this particular purpose include cherries, blueberries, carrots, bananas, beetroot,

blackcurrants, pomegranates, pears, etc. (Howatson *et al.*, 2010; Wootton-Beard *et al.*, 2011; Lamprecht, 2015; Nieman *et al.*, 2015; Clifford *et al.*, 2017a; Clifford *et al.*, 2017b; Hurst *et al.*, 2019). Moreover, it has been proposed that the anti-oxidant and anti-inflammatory capacity of beetroot (*Beta vulgaris L.*) surpasses that of most other fruits and vegetables due to its abundant betalain, among other phytochemicals (polyphenols, vitamin C, rutin, epicatechin, etc.) content, which inhibits cyclooxygenase activity and disrupts nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Hadipour *et al.*, 2020). In addition to these pigment imposing betalains, beetroot is also considered a Class A performance-enhancing supplement (supported by adequate scientific research) by the Australian Institute of Sport (2019) due to its nitrate donating abilities. Nitrate reportedly enhances NO bioavailability via the nitrate-nitrite-NO pathway, subsequently enhancing vasodilation, cellular respiration regulation and neurotransmission (Lundberg *et al.*, 2008; Jonvik *et al.*, 2015; Clifford *et al.*, 2017a). Besides the successful application of beetroot juice as a performance-enhancing supplement, controversial evidence exists regarding its ability to expedite the physiological recovery process after exercise (Clifford *et al.*, 2017a; Clifford *et al.*, 2017c). To our knowledge, no study has investigated the capacity of beetroot juice to facilitate metabolic recovery after ER.

Thus, in this double-blinded, placebo-controlled investigation, an untargeted GCxGC-TOFMS metabolomics approach was used to determine whether or not beetroot juice ingestion possesses an added advantage towards metabolic recovery when compared to unaided recovery within 48 h post-marathon. This knowledge could not only further improve the current understanding of beetroot aided recovery, but also provide clues to new, more effective dose-dependent supplementation beetroot ingestion after exercise.

6.3. METHODS AND MATERIALS

Considering that this project forms part of a larger study that consists of multiple (inter-disciplinary) aims, additional clinical and physiological measurements (Clifford *et al.*, 2017a), as well as complementary metabolic investigations (Stander *et al.*, 2018; Stander *et al.*, 2020a) based on sub-divisions of the current cohort, have already been published and may be referred to for additional information not pertinent to this investigation.

6.3.1. Participants and clinical samples

Since this Chapter is aimed at determining whether or not beetroot juice may expedite the metabolic recovery process within 48 h post-marathon, all the serum samples of both the placebo (n=16; 10 males and 6 females) and beetroot juice-ingesting athletes (n=15; 9 males and 6 females) were included in this part of the investigation (Figure 3-1). In addition to the aforementioned overview of the participant selection and/or eligibility criteria (Section 2.4), marathon layout (Section 2.5), as well as sample collection and pre-processing procedures (Section 2.6), supplement particulars such as time and frequency of ingestion (Section 2.5) and the macro-nutrient composition of these (Table 3-2) were also previously discussed in Chapter 3. Besides these

recovery modality restrictions, the use of anti-bacterial mouth wash was prohibited as a means of conserving the proposed bacterial nitrate-nitrite conversion in the mouth. Withal, Table 3-1 (Chapter 3) contains a detailed summary of the characteristics of the respective cohorts and for the purpose of brevity, will not be described again in this section.

6.3.2. Sample analysis

As previously comprehensively discussed (Chapter 3), preparation of serum samples commenced with an in-house total metabolome extraction and derivatisation protocol (Section 2.8), before being injected into the Pegasus 4D GCxGC-TOFMS for analysis (Section 2.9). The data generated from this analytical apparatus was then processed (deconvolution, peak alignment and identification) using ChromaTOF software (Section 3.2.8).

6.3.3. Data processing and statistical analyses

Similar data processing methods were employed as described in Chapter 3 and 4, however, for this section a 50% QC-CV filter was utilised, thus retaining metabolites with a $CV \leq 50\%$.

Following these clean-up steps, the data was subjected to a variety of multivariate and univariate statistical methods using MATLAB (MATLAB, 2012) (in conjunction with a PLS (Eigenvector Research, 2016) toolbox), as a means of selecting those metabolites pertinent to the aim of this investigation. In order to comprehensively address the aim of this investigation, multiple statistical objectives, and therefore comparisons, were required. In summary, paired statistical analysis of the beetroot juice-ingesting cohort was performed to confirm whether this cohort indeed recovered to a pre-marathon-related state within 48 h (statistical objective A), as has already been confirmed for the placebo group (Stander *et al.*, 2020a). Hence, the 24 h and 48 h post-marathon serum metabolite profiles of the beetroot-ingesting cohort were respectively compared to that of the pre-marathon profile to identify any differentiating metabolites which would oppose metabolic recovery. Multivariate analyses included multilevel (ML) PCA and ML-PLS-DA, while univariate analyses consisted of a paired t-test, and effect size tests, to assess statistical and practical significance respectively. To control for FDRs associated with large scale multiple testing, as in the case of untargeted metabolomics datasets, t-test *p*-values were adjusted using the Benjamini-Hochberg procedure (limiting FDRs to 5%). All metabolites (pre-marathon vs 24 h post-marathon and pre-marathon vs 48 h post-marathon) with a BH adjusted *p*-value ≤ 0.05 and an effect size *d*-value ≥ 0.5 were deemed significant.

Furthermore, in order to determine whether or not beetroot juice ingestion expedites the metabolic recovery trend of athletes within 48 h post-marathon, unpaired statistical analyses of the beetroot cohort vs placebo cohort were performed (statistical objective B). Foremost, the metabolic progression for both treatment groups over time was compared by using statistical models that accounted for the entire experimental design and dependencies between measures, i.e. a two-way repeated-measures analysis of variance (RM ANOVA), and

an unfolded PCA. The latter transforms a three-dimensional tensor into a two-dimensional matrix (Figure B-1 in Annexure B), thus allowing for PCA (Villez *et al.*, 2009). To supplement these comprehensive statistical methods, recovery time-point-specific inter-cohort comparisons were performed to assess day-specific variation that may be apparent between the cohorts. For this, multivariate statistical methods included PCA, PLS-DA, whilst univariate methods comprised an independent samples t-test (FDR's controlled to 5% according to the BH procedure), and independent effect size tests based on Cohen's *d*-values. Here, variables were selected based on a BH adjusted *p*-value ≤ 0.05 or a Cohen's *d*-value ≥ 1.0 , thus considering both statistical and practical significance. However, as opposed to the equal statistical and practical relevance of marker selection in objective A, this objective's selection was more stringent on practical relevance to capture slight differences that may be of practical importance.

Since the cohort sizes of this investigation are relatively small, multivariate models are less readily validated and could therefore only be used to visualise trends and variation, whilst univariate models, better equipped to avoid false discoveries, were employed for variable selection in both statistical objectives.

6.4. RESULTS

When comparing both the 24 h and 48 h post-marathon serum metabolite profiles of the beetroot cohort to the corresponding pre-marathon profiles (statistical objective A), no metabolite markers were considered statistically significant (BH *p*-value ≤ 0.05 and *d*-value ≥ 0.5), suggesting that the metabolome of the beetroot cohort recovered to a baseline-related state within 48 h post-marathon. This was further indicated by the confined positioning of the respective ML-PCA of each recovery time-point relative to the pre-marathon ellipsoids, as presented in Figure 6-1.

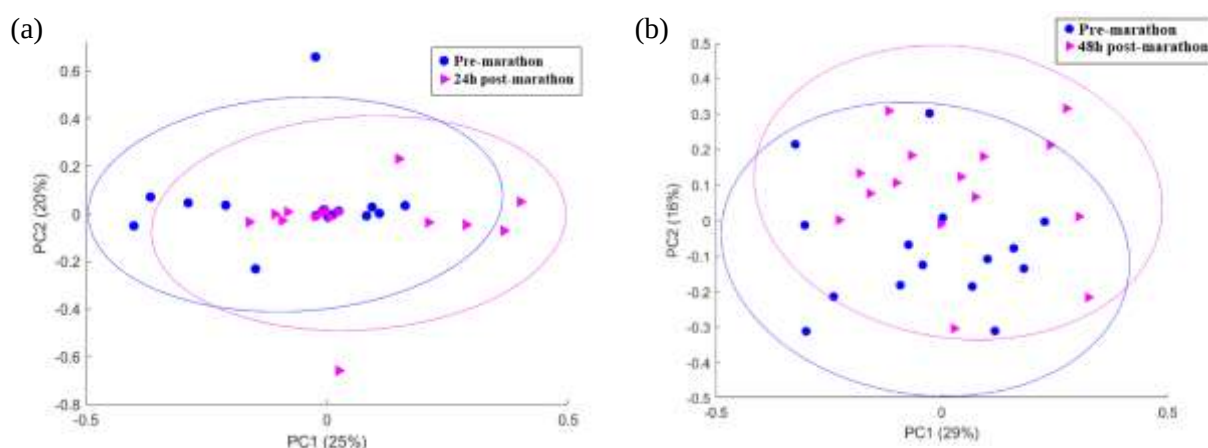


Figure 6-1: Multi-level principal component analysis plot of the pre-marathon serum metabolite profiles of the beetroot juice group relative to the (a) 24h post-marathon and (b) 48h post-marathon metabolite profiles of the same group. Abbreviations: PC: principal component.

Since these results correspond with the findings of Stander *et al.* (2020a) that indicated overall metabolic restoration of the placebo athletes within 48 h post-marathon, an unfolded PCA was constructed to visualise the time-dependent global metabolic recovery trend of the two cohorts relative to each other (Figure 6-2) (statistical objective B).

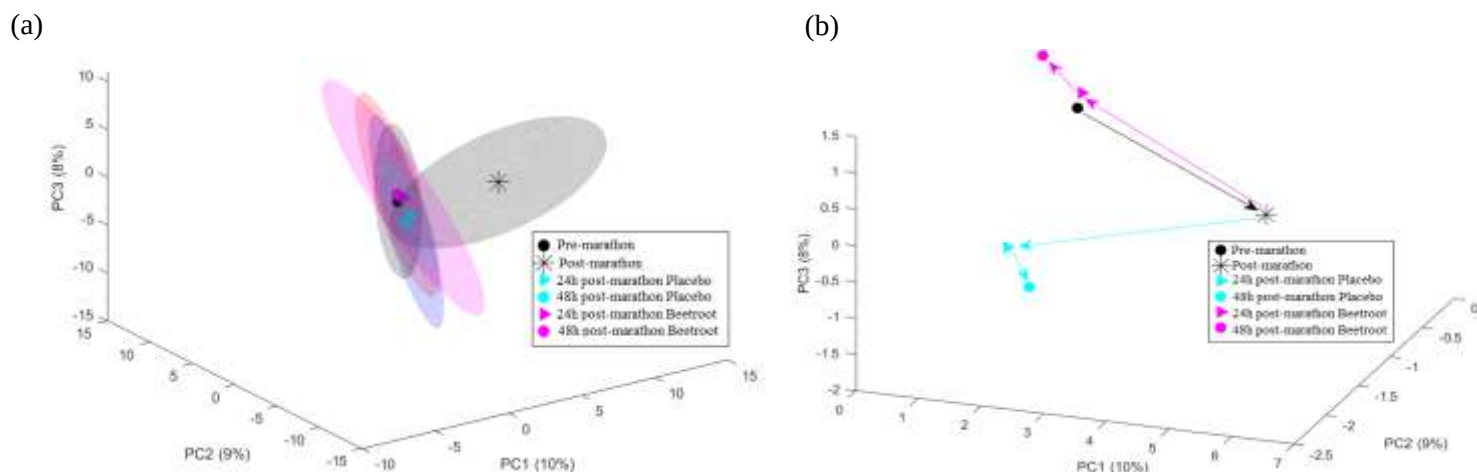


Figure 6-2: Unfolded principal component analysis (a) with, and (b) without confidence intervals, depicting the global recovery trend of the beetroot and placebo cohort over time. Abbreviations: PC: principal component.

From this, it is evident that the most significant effect emanated from the marathon perturbation (Stander *et al.*, 2018), after which both groups returned to a pre-marathon-related state within ± 24 h (Stander *et al.*, 2020a). Intensely overlapped confidence intervals of the recovery time-points of the beetroot juice and placebo cohorts suggest little to no variation between groups at these times (Figure 6-2a). However, upon removal of confidence intervals, 24 h and 48 h post-marathon centroids of the beetroot juice-ingesting cohort are seemingly slightly closer to that of the pre-marathon (Figure 6-2b), which could be indicative of small inter-cohort differences. As such, a two-way RM ANOVA was employed, and 16 metabolites were identified as having a statistically significant (p -value ≤ 0.05) interaction (between time and intervention) and/or intervention effect (Table 6-1). Considering that this univariate method focuses on relatively small differences within the entire study design, the RM ANOVA alone does not discriminate the precise origin of the metabolic fluctuation observed. Consequently, pairwise comparisons of the different time-points were employed to determine day-specific recovery effects. Whilst the PCA plots of each time-point comparison (Figure B-2 in Annexure B) did not visually portray significant differences between the groups at the different time-points, Cohen's d -values for the majority of these RM ANOVA metabolites indicated random, moderate-to-large practical variance amongst the groups (Table 6-1). A more detailed table including RM ANOVA p -values as well as average metabolite concentrations and standard deviations can be found in Table B-1.

Table 6-1: Metabolites varying significantly between the beetroot and placebo cohorts, according to RM ANOVA and day-specific pairwise comparisons.

Metabolite (PubChem ID)	Pre-marathon Placebo vs Beetroot juice		Post-marathon Placebo vs Beetroot juice		24 h post- marathon Placebo vs Beetroot juice		48 h post- marathon Placebo vs Beetroot juice	
	BH p-value	Cohen's d-value	BH p-value	Cohen's d-value	BH p-value	Cohen's d-value	BH p-value	Cohen's d-value
α -Oleoylglycerol (5319879) ^b	0.89	0.65	0.92	0.31	1.00	0.13	0.89	0.19
β -Hydroxydecenedioate (121232666) ^b	0.92	0.33	0.97	0.05	1.00	0.37	0.56	0.44
β -Hydroxyphenylacetate (12122) ^a	0.97	0.22	0.92	0.60	1.00	0.34	0.27	0.85
β -Hydroxyvalerate (107802) ^a	0.92	0.33	0.92	0.54	1.00	0.58	0.31	0.64
<i>p</i> -Hydroxyphenylacetate (127) ^a	0.89	0.61	0.92	0.31	1.00	0.65	0.30	0.70
Arabitol (43925) ^{a,b,*}	0.89	0.45	0.96	0.12	0.03	1.40	2.1x10 ⁻³	1.73
Eicosanoate (10467) ^a	0.89	0.58	0.92	0.46	1.00	0.36	0.50	0.56
Glycerol (753) ^b	0.97	0.13	0.92	0.57	1.00	0.24	0.99	0.02
Hippurate (464) ^a	0.89	0.59	0.92	0.39	1.00	0.19	0.27	0.86
Mannitol (6251) ^a	0.99	0.11	0.92	0.37	1.00	0.55	0.37	0.59
Nonanoate (8158) ^{a,*}	0.89	0.71	0.92	0.84	0.57	1.02	2.6x10 ⁻³	1.63
Oxalate (971) ^a	0.89	0.40	0.92	0.79	1.00	0.49	0.92	0.12
Rhamnose (25310)	0.89	0.63	0.92	0.46	1.00	0.65	0.88	0.22
Threonate (21145021) ^{a,b}	0.88	0.61	0.89	1.03	1.00	0.30	0.52	0.58
Undecanoate (8180) ^{a,*}	0.40	1.24	0.92	1.07	1.00	0.35	0.04	1.38
Xylose (135191) ^{a,b,*}	0.89	0.56	0.92	0.29	0.21	1.04	0.53	0.46

Footnote: Intervention (a), interaction (b) effects, and day-specific significance (*) are indicated in the first column, while day-specific p- and d-value significance is indicated with grey shading.

In light of the pre-existing individual variance (particularly post-marathon), as well as the lack in statistical significance of metabolites (BH *p*-value ≤ 0.05) during the recovery period (24 h and 48 h post-marathon), only those metabolites with a large effect (*d*-value ≥ 1.0) at the respective recovery time-points were considered relevant in terms of understanding any possible treatment-induced differences at various stages of recovery (24 h and 48 h post-marathon), when comparing the beetroot and placebo cohort. Based on this, only four metabolites (arabitol, nonanoate, undecanoate and xylose) were identified as significantly altered when comparing these cohorts (indicated with an asterisk in Table 6-1), while significant *p*- and *d*-values are emphasized by grey shading.

6.5. DISCUSSION

Beetroot juice has gained a considerable amount of attention as a possible performance optimisation and recovery enhancing sports supplement, due to its potent reactive oxygen scavenging properties and its ability to promote vasodilation, regulate cellular respiration and reduce inflammation (Hadipour *et al.*, 2020; Mirmiran *et al.*, 2020). However, limited literature is available pertaining to the metabolic recovery effects of beetroot juice after ER exercises. In order to completely ascertain whether or not beetroot juice ingestion possess an added advantage in terms of metabolic recovery within 48 h post-marathon, it is imperative to also comprehend the unaided metabolic recovery of athletes within the same timeframe. These results have been published as part of a previous investigation (Stander *et al.*, 2020a), and will therefore only be summarised here. Unaided metabolic recovery was attained within 24 h post-marathon (with exception of xylose), and included reductions in carbohydrates, fatty acids and TCA cycle intermediates, along with elevations in amino acids. Recovery of these was ascribed to a reduction in energy requirements, leading to the activation of glycogenesis, fatty acid re-esterification, protein synthesis, cellular membrane restoration and the possible activation of nucleotide salvage pathways (Nieman *et al.*, 2014; Stander *et al.*, 2020a). In this investigation, a comprehensive comparison of the recovery trends of the beetroot juice and placebo cohorts did not vary significantly (Figure 6-2), thus indicating no added advantage of beetroot juice ingestion in terms of metabolic recovery after the marathon. However, when employing a two-way RM ANOVA, significant (albeit small) concentration fluctuations were observed in 16 metabolites (Table 6-1). Whilst these fluctuations were significant when considering the entire study design, pairwise analysis of the respective time-points later revealed that a large portion is owed to post-marathon (and to some extent pre-marathon) variation between athletes. Besides the inevitable confounder of genetic, age and gender variation, these post-marathon disparities are to be expected since the dietary regimens of athletes were not restricted and/or controlled at either of these time-points as a means of providing a more relatable, robust representation of the marathon perturbation (Stander *et al.*, 2018). Regardless, while the substantial impact of the marathon perturbation totally overshadowed these differences (Stander *et al.*, 2018), the recovery effects of the beetroot juice intervention are evidently negligible on the metabolic level, thus supporting previous literature (Betteridge *et al.*, 2016; Clifford *et al.*, 2017a). As such, it is unsurprising that only four metabolite markers were identified to vary significantly at the different stages of recovery (24 h post-marathon and 48 h post-marathon). These metabolites (arabitol, xylose, nonanoate, and undecanoate) will be comprehensively discussed below.

In addition to the initial increase of arabitol in both cohorts during the marathon (Stander *et al.*, 2018), this metabolite continued to increase in the beetroot-ingesting cohort during the recovery period, while contrarily decreasing in the placebo cohort (Stander *et al.*, 2020a). Although at first glance this may be associated with low-calorie sweetener ingestion, as previously reported to occur during the marathon (Stander *et al.*, 2018), *Love Beets* product labels indicated that no additional sweeteners were added to this 99% organic beetroot juice supplement (with 1% lemon juice as a stabiliser). However, considering that red beetroot pectin fibres

are rich in various sugars including xylose, rhamnose, mannose and especially, arabinose (Lim, 2016), it can be deduced that although not directly added to the supplement, arabitol may have been produced via the reduction of arabinose in the polyol pathway present in humans and various intestinal yeasts (Kumdam *et al.*, 2014; Kordowska-Wiater, 2015). Furthermore, it has been reported that intestinal bacteria preferentially ferment arabinose over other sugars such as xylose (Desai & Rao, 2010), thus further ascribing the elevated xylose bioavailability 24 h post-marathon in this cohort. In addition, xylose can serve as a minor precursor for ribose, the latter of which is a key component in the uridine-dependent salvage pathway during recovery (Stander *et al.*, 2020a). As such, since beetroot phytonutrients, including betaines (Lane & Fan, 2015), folate (Puigserver, 2018), and glutamine (Lim, 2016), can assist in the process of nucleotide restoration, the observed 24 h post-marathon elevations in xylose could also partially be indicative of the role of beetroot-promoting nucleotide synthesis via alternative constituents, subsequently reducing the incorporation of xylose to produce ribose. Nevertheless, whilst still being significantly elevated compared to the placebo cohort, slight 48 h post-marathon reductions (compared to 24 h post-marathon) in both arabitol (significant) and xylose (non-significant) were observed in the beetroot juice cohort and may be attributed to the fact that only a single beetroot supplement (upon waking) was ingested on the final day of the investigation (48 h post-marathon), as opposed to the three servings immediately post-marathon and 24 h post-marathon. Nevertheless, arabitol has been associated with numerous health benefits, of which the prevention of blood sugar dysregulation and fat deposition in the intestinal tract (Kordowska-Wiater, 2015) is the most applicable to post-race metabolic recovery.

Furthermore, opposing fluctuations in OCFA (nonanoate and undecanoate) concentrations were observed during recovery. In general, reductions in undecanoate (C₁₁) observed in both cohorts within 24 h post-marathon, is most likely due to residual catabolism of the accumulated fatty acids via α -oxidation (Stander *et al.*, 2018). This is further supported by elevations in nonanoate (C₉) in both cohorts during this time since it is a downstream product of undecanoate catabolism. Nevertheless, mitigation of OCFA catabolism is evident by the reduction in both nonanoate and undecanoate concentrations in the placebo cohort within 48 h post-marathon. Contrarily, however, both of these OCFA are elevated in the beetroot cohort 48 h post-marathon, possibly indicating the activation of OCFA synthesis in this cohort. The proposed mechanism of action for this includes the fermentation of beetroot sugar-content to propanoate via intestinal bacteria (*Bacteroidetes*, *Clostridium*, *Lactobacillus*, *etc.*) (Larsen *et al.*, 2019), which can be further utilised for OCFA synthesis. Argumentatively, placebo supplements evidently contained the same total amount of sugar, however, this did not correlate an increase in OCFA 48 h post-marathon. This may not only be ascribed to the increased propionate-producing bacterial activity (i.e. *Lactobacillus*) induced by beetroot pectin ingestion (Larsen *et al.*, 2019), but may also arise as a result of the polyphenol content in beetroot which promotes the ability to reduce effective carbohydrate absorption in the intestines (Wootton-Beard *et al.*, 2014), subsequently prolonging the exposure of beetroot sugars to gut microbes for fermentation. Besides the possible contribution of OCFA to phospholipid content (i.e. cellular membrane restoration), recent findings have shown an inversed correlation

of OCFA and leptin, as well as plasminogen-inhibitor-1 (Kurotani *et al.*, 2017), further suggesting its role in energy homeostasis regulation and the activation of fibrinolysis. Nevertheless, whether or not elevated OCFA has an additional advantage pertaining to athlete recovery, remains to be determined.

6.6. CONCLUSION

This investigation not only supported previous findings of total metabolic recovery of athletes within 48 h post-marathon, but further indicated that beetroot juice ingestion does not significantly expedite the metabolic recovery process induced by endurance exercise. Although several significant, albeit small, metabolic fluctuations between cohorts were selected based on univariate statistics, the majority revealed considerable random variation immediately post-marathon, which is owed to opposing athletic regimens and inherent biological and physiological aspects. As such, only metabolites with large practical significance at the respective days of the supplement interventions (24 h and 48 h post-marathon) were considered when addressing the aim of this investigation. Of the four metabolites identified, the majority were associated with the glycaemic content of red beetroot, as well as the increased microbial activity stimulated by this vegetable. Whether or not these may provide an additional advantage during an extended period of recovery remains to be elucidated. However, the lack of beetroot juice effectiveness may stem from dose-dependent absorption of phytonutrients central to the recovery process, and the considerable dependence on the composition and fermentation preferences of gut-microbes.

6.7. LIMITATIONS AND FUTURE PROSPECTS

As with all human-based investigations, an inevitable limitation includes human genotype/phenotype (sex, age, and ethnicity [latter not recorded]) variation confounders, as well as possible dietary variation amongst athletes. Considering the list of restrictions provided to the athletes in terms of recovery modalities and prohibition of anti-bacterial mouth wash, additional dietary restrictions would have potentially imposed on personalised regimens of these athletes, leading to a reduction in the already confined cohort size. Nevertheless, although not controlled, dietary intake was recorded for the entire duration of this investigation in an attempt to circumvent this. However, these individual variations may introduce a higher level of robustness into the results, providing a more realistic and relatable representation of the metabolic effects of incremental beetroot juice supplementation after a marathon. Since the cohort size of this investigation is considered relatively small in terms of human-based studies and the intricate accompanying statistical approaches needed for analysing the data, future studies consisting of a larger cohort (particularly in terms of sex), could further validate these findings and possibly determine the metabolic effects of beetroot juice in a gender-dependent manner. Additionally, the lack in the efficacy of beetroot juice observed in this study may be due to the rapid restoration of the metabolism even when not subjected to aiding strategies, therefore future studies may consider observing the effect(s) of beetroot supplements at shorter incremental time-points along this recovery process (i.e. 6 h, 12 h, 18 h, etc.). Furthermore, since the microbiome of athletes seems to be a

key component in the conversion of red beetroot nitrate, the fermentation of sugar components, and metabolism of polyphenols, metabolic studies investigating the effects of beetroot on the GM and microbial function could determine the fermentation preferences and possible causes for metabolic delays pertinent to phytonutrient uptake.

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CHAPTER 7: CONCLUSION AND FUTURE PROSPECTS

Considering that the results have been comprehensively discussed in the relevant chapters, this chapter will merely provide an overview of the key findings and concluding remarks as it relates to the overall scope of this investigation. Succeeding this, possible future prospects emanating from this study will also be provided and briefly discussed.

7.1. CONCLUDING REMARKS

Besides the well-described physiological and immunological effects of ER, limited knowledge exists pertaining to endurance race-induced metabolic adaptations, and the subsequent recovery thereof. Since metabolomics allows for the identification and quantification of metabolite fluctuations in response to an external and/or internal stimuli, as well as the fact that serum is the primary liquid carrier of most metabolites, serum metabolomics is a formidable tool that may assist in filling this current void. As such, this approach was applied in this study, to not only provide a holistic representation of the metabolic adaptations induced by a marathon, but also to elucidate the recovery trend of these metabolites within 48 h post-race, with and without the ingestion of beetroot juice as a potential cost-effective recovery supplement.

Pairwise comparisons of the pre- and immediate post-marathon serum metabolite profiles of athletes indicated an unequivocal metabolic shift, as a result of the extensive energy taxing nature of this perturbation. As such, the majority of the 70 metabolite markers best describing the variation between the metabolic states were directly associated with various fuel substrate catabolism pathways (i.e. glycolysis, lipolysis, and AA oxidation), gluconeogenesis, ketogenesis, and possibly the hampering of insulin secretion. Elevations in β - and α -hydroxy acids along with OCFA not only indicated the activation of alternative FA catabolism pathways due to an imbalanced redox potential and mechanistic FA oxidation component saturation, but also the activation of autophagy as a means of releasing additional embedded fuel substrates. Withal, some of these metabolite fluctuations were also attributed to elevated oxidative stress, activation of mechanisms opposing ROS production, as well as liver/skeletal muscle damage.

However, when construing the unaided metabolic recovery profiles of the placebo cohort, it was clear that these metabolic adaptations recovered to baseline-related concentrations within 24 h post-marathon, with the exception of dietary xylose. Significant metabolic fluctuations (61 metabolites) were primarily ascribed to momentary catabolism of residual fuel substrates, followed by the activation of anabolic processes such as glycogenesis and FA re-esterification as a result of a reduction in energy requirement, and the restoration of the intracellular glycaemic flux. Moreover, corresponding uridine and ribose fluctuations 24 h and 48 h post-race postulated the activation of nucleotide salvage pathways and/or the restoration of damaged cellular membranes.

Based on this, the metabolic profiles of the beetroot juice-ingesting cohort were compared to that of the placebo cohort to determine if beetroot may aid in expediting metabolic recovery within 48 h post-race. Whilst a comprehensive comparison of the recovery trends of the cohorts showed little to no variation, significant (yet small) metabolic fluctuations were identified when employing sensitive univariate statistics. However, upon further investigation, considerable random inter-cohort variation was apparent immediately post-marathon due to opposing athlete regimens and inherent biological factors. Thus, only metabolites of high practical significance within the recovery period were considered a good representation of the efficacy of beetroot juice as a recovery agent. To this end, only four metabolites were identified, and were either direct or secondary metabolites of beetroot constituents and/or microbial fermentation thereof. Hence, it is clear that the effects of the beetroot juice intervention on metabolic recovery seem so diminutive that it is overshadowed by any natural, random variation. Nevertheless, impeded beetroot juice effectiveness may stem from dose-dependent absorption of phytonutrients, and the considerable dependence on the composition and fermentation preferences of the microbiome

An integrated view of these results suggests that although a marathon perturbation has a profound effect on the human metabolome, the metabolic flexibility of athletes allow for an expeditious metabolic recovery regardless of the assistance of cost-effective, performance and health-promoting supplements such as beetroot juice.

7.2. FUTURE PROSPECTS

- Since this investigation is one of the first to report elevated OCFA immediately after an ER race, more targeted approaches (i.e. lipidomics) may elaborate on the precise origin and functionality of these in the current setting.
- Considering that the majority of the metabolic adaptations immediately post-race is centred on the utilisation of various fuel substrates, it may be interesting to observe the metabolic variance in subsets of athletes ingesting comparable amounts of either carbohydrates, lipids or AAs for a pre-determined time preceding a marathon. These metabolic profiles may then be correlated with markers for cellular autophagy, muscle damage, insulin secretion, and/or athletic performance.
- As suggested in Chapters 5 and 6, future studies consisting of larger cohorts and/or serum samples obtained at additional time-points after the marathon may not only further validate the findings presented in this investigation, but could also allow for better metabolic mapping of the in-between stages of metabolic recovery, leading to the identification of additional recovery-specific metabolic markers.

- Since metabolic recovery seems to be attained as early as 24 h post-marathon (without the assistance of specific recovery strategies) it may be interesting to investigate the effects of continuous beetroot juice ingestion after more energy-intensive exercises (i.e. triathlon) or races of greater distances (≥ 100 km), while further adjusting the dosage of beetroot juice ingestion.
- Considering the role of the microbiome in the context of the digestion, absorption and bioconversion of beetroot juice constituents, it may be worth determining the effects of endurance races on the composition of microbial species prevalence, as well as the influence of a combination of probiotics and beetroot ingestion at various time points before, during and after endurance training.

ANNEXURE A

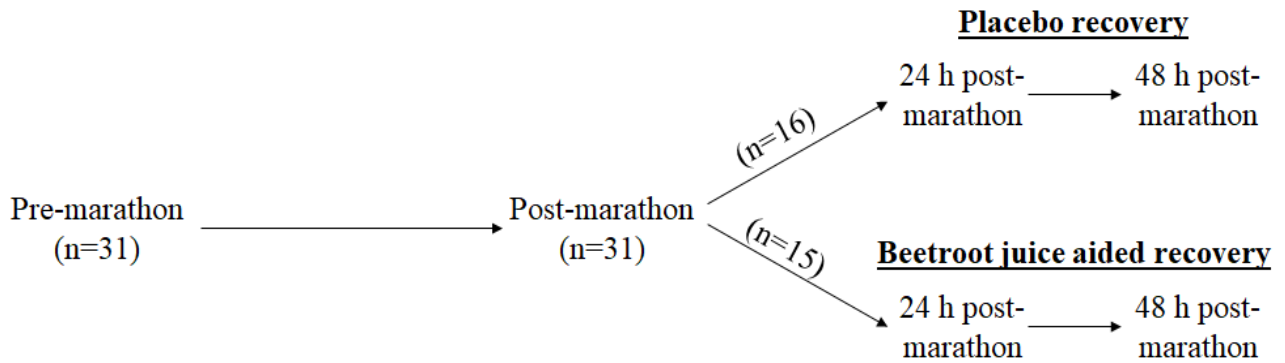
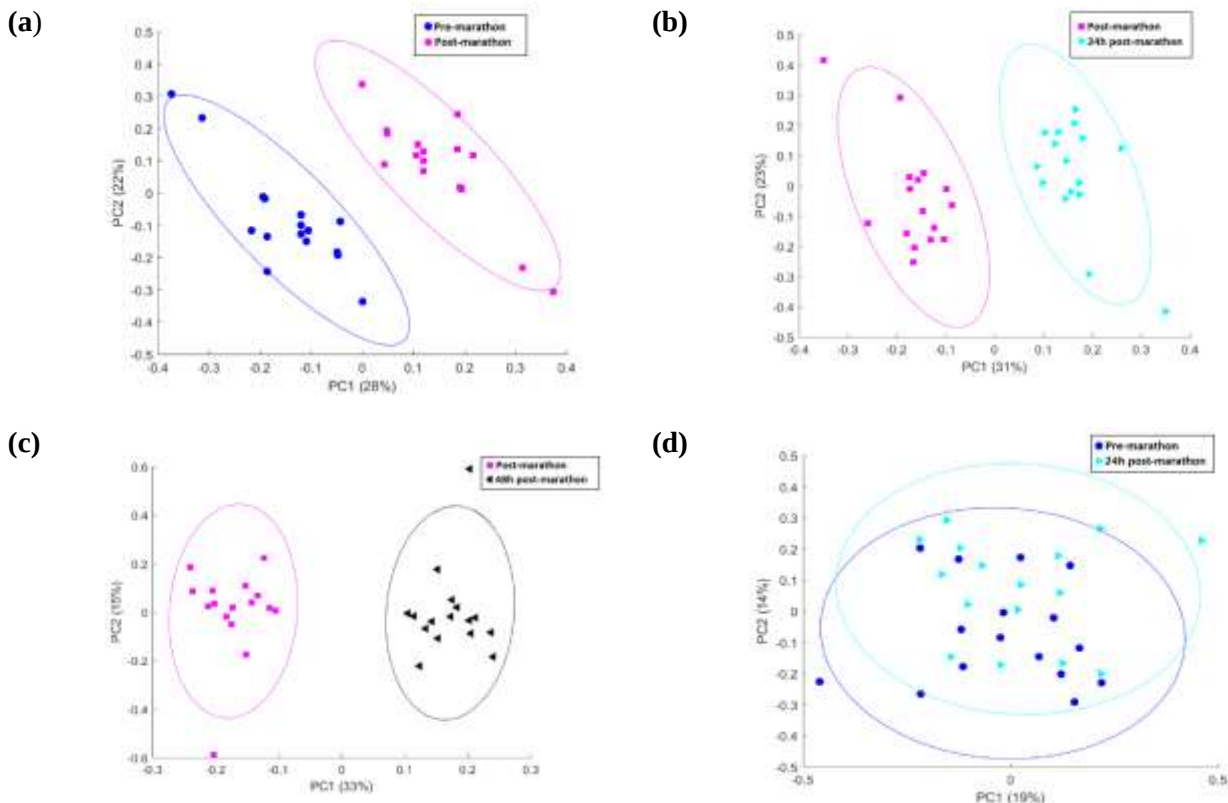


Figure A-1: The larger scope of this investigation consists of multiple objectives: Objective 1 (Chapter 4): Effects of a marathon on the serum metabolome of athletes (n=31); Objective 2 (Chapter 5): Metabolic recovery without the intervention of recovery aids, by comparing pre-, post- as well as 24 h and 48 h post-marathon samples of the athletes that ingested placebo supplements (n=16); Objective 3 (Chapter 6): Effect of beetroot juice supplementation on metabolic recovery, by comparing pre-, post- as well as 24 h and 48 h post-marathon samples of the athletes that ingested beetroot juice supplements (n=15).



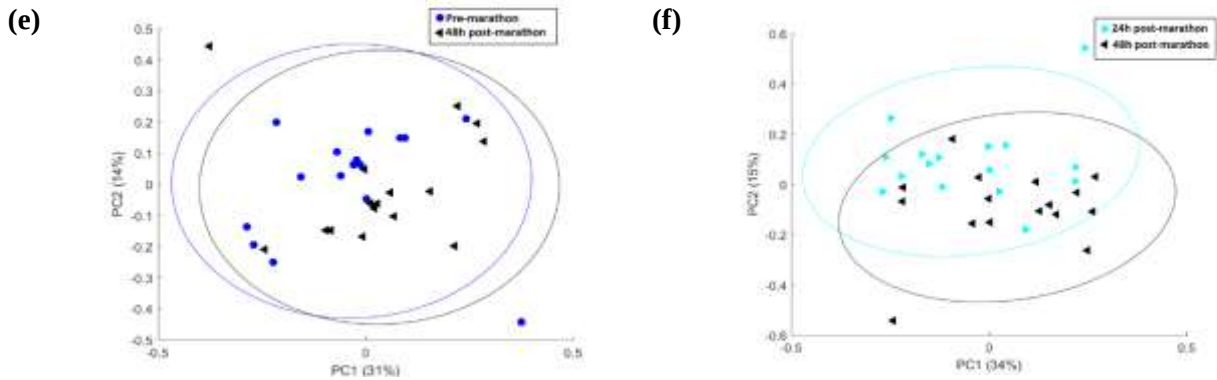
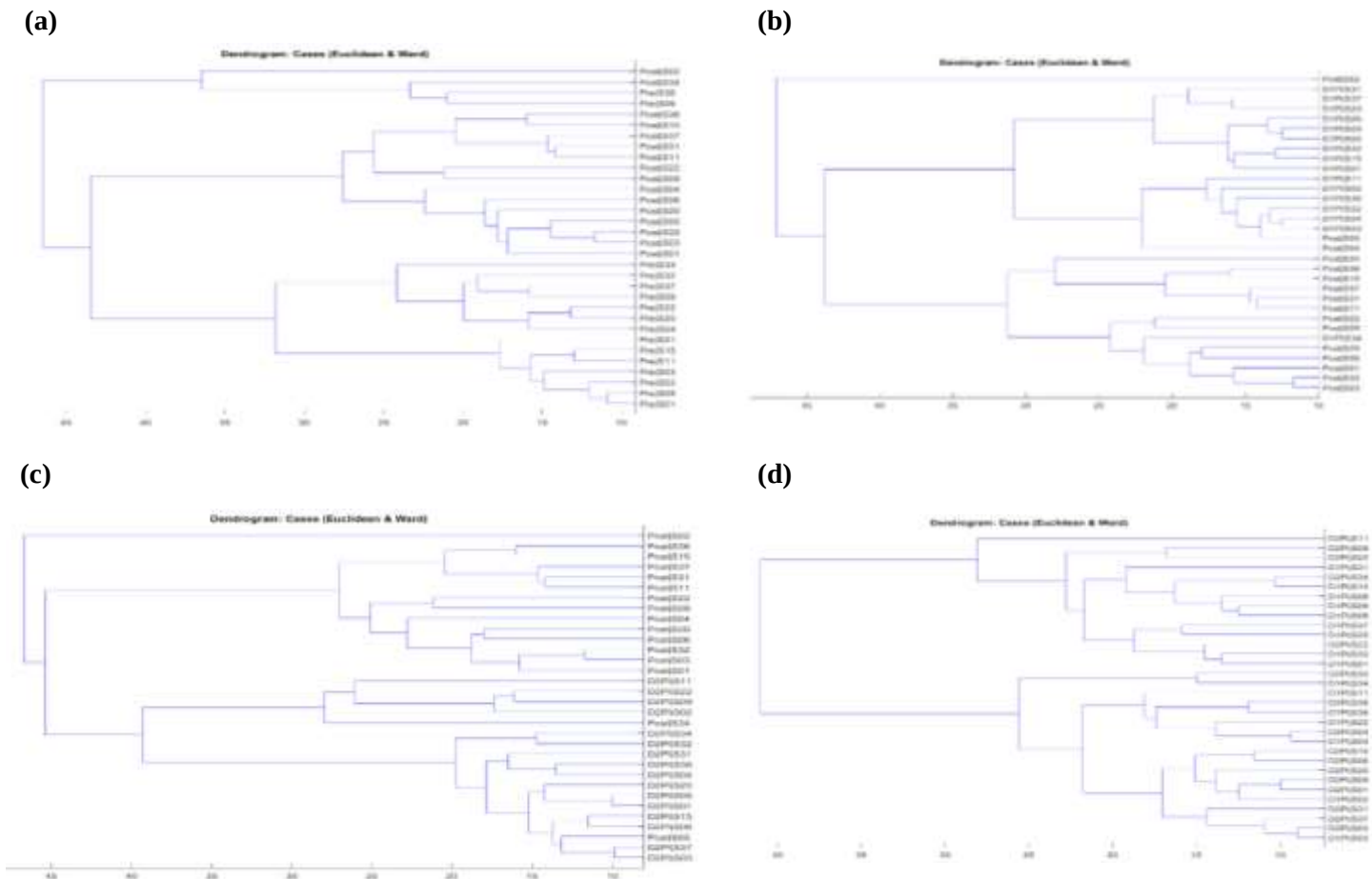


Figure A-2: Multilevel principal component analysis plots of the comparative groups: (a) Pre-marathon (denoted by blue/circle) vs post-marathon (denoted by pink/square); (b) post-marathon (denoted by pink/square) vs 24 h post-marathon (denoted by turquoise/triangle); (c) post-marathon (denoted by pink/square) vs 48 h post-marathon (denoted by black/triangle); (d) pre-marathon (denoted by blue/circle) vs 24 h post-marathon (denoted by turquoise/triangle); (e) pre-marathon (denoted by blue/circle) vs 48 h post-marathon (denoted by black/triangle); (f) 24 h post-marathon (denoted by turquoise/right-tilted triangle) vs 48 h post-marathon (denoted by black/left-tilted triangle). Abbreviations: PC: principal component.



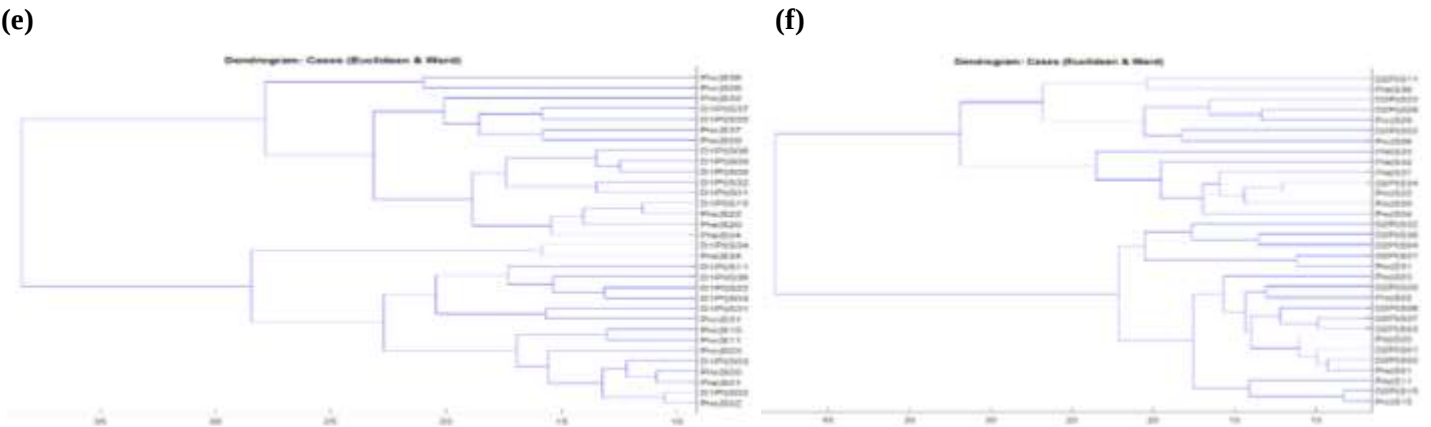


Figure A-3: Dendrogram metabolome clustering of athletes at the various comparative timepoints: (a) Pre-marathon vs post-marathon, (b) post-marathon vs 24 h post-marathon, (c) post-marathon vs 48 h post-marathon, (d) 24 h vs 48 h post-marathon.

Table A-1: Participant characteristic and performance distribution in relation to the entire cohort.

Distribution count and frequency		Criteria						
Age distribution (yrs)								
		20-30	30-40	40-50	50-60	60-70		
Count		3	4	7	0	2		
Frequency		19%	25%	44%	0%	13%		
Gender								
		Female			Male			
Count		6			10			
Frequency		38%			63%			
Marathon performance (duration)								
		3h00–3h30	3h30–4h00	4h00–4h30	4h30–5h00	5h00–5h30	5h30–6h00	6h00–6h30
Count		2	5	2	2	2	2	1
Frequency		13%	31%	13%	13%	13%	13%	6%

Table A-2: Serum metabolite markers (n=61) best describing the detected variation and metabolic recovery of the athletes within 48 h post-marathon.

Compound name (PubChem ID)	Pre-marathon	Post-marathon	24 h post-marathon	48 h post-marathon	Pre- vs post-marathon		Post- vs 24 h post-marathon		Post- vs 48 h post-marathon		24 h vs 48 h post-marathon		Pre- vs 24 h post-marathon		Pre- vs 48 h post-marathon	
	Average concentration (µg/mL) (standard deviation)				Corrected <i>p</i> -value	<i>d</i> -value	Corrected <i>p</i> -value	<i>d</i> -value	Corrected <i>p</i> -value	<i>d</i> -value	Corrected <i>p</i> -value	<i>d</i> -value	Corrected <i>p</i> -value	<i>d</i> -value	Corrected <i>p</i> -value	<i>d</i> -value
	Carbohydrate and associated intermediates															
Arabitol (439255)	0.004 (0.001)	0.005 (0.002)	0.004 (0.001)	0.003 (0.001)	0.005	1.072	0.001	0.963	1.0x10 ⁻⁴	1.346	0.392	0.414	0.904	0.049	0.949	0.358
Erythritol (222285)	0.007 (0.003)	0.012 (0.004)	0.008 (0.006)	0.005 (0.002)	7.8x10 ⁻⁶	1.687	0.003	1.732	4.6x10 ⁻⁶	2.811	0.392	0.734	0.970	0.027	0.091	1.095
Glucose (5793)	4.7x10 ⁻⁴ (3.6x10 ⁻⁴)	0.004 (0.007)	0.001 (0.001)	3.6x10 ⁻⁴ (3.1x10 ⁻⁴)	0.001	9.003	0.004	1.107	0.001	1.251	0.392	0.663	0.786	0.913	0.949	0.365
Mannitol (6251)	0.003 (0.004)	0.005 (0.004)	0.004 (0.003)	0.003 (0.003)	0.075	0.529	0.313	0.488	0.001	0.955	0.508	0.401	0.842	0.140	0.949	0.232
Mannose (18950)	2.2x10 ⁻⁴ (1.3x10 ⁻⁴)	0.003 (0.007)	3.6x10 ⁻⁴ (3.6x10 ⁻⁴)	1.8x10 ⁻⁴ (1.4x10 ⁻⁴)	0.001	9.688	0.004	1.107	0.001	1.261	0.392	0.554	0.786	0.915	0.949	0.308
Myo-inositol (440194)	0.031 (0.037)	0.035 (0.013)	0.026 (0.007)	0.022 (0.004)	0.234	0.486	0.028	0.691	0.000	1.165	0.311	0.580	0.929	0.043	0.949	0.261
Psicose (441036)	0.012 (0.022)	0.014 (0.006)	0.006 (0.003)	0.010 (0.011)	0.083	0.638	0.004	1.479	0.127	1.057	0.575	0.491	0.794	0.238	1.000	0.012
Rhamnose (5460029)	0.001 (0.001)	0.001 (0.001)	0.001 (0.001)	0.001 (4.9×10 ⁻⁴)	0.043	1.081	0.110	0.742	0.001	1.201	0.398	0.520	0.849	0.170	0.949	0.395
Ribose (10975657)	0.013 (0.015)	0.015 (0.007)	0.009 (0.003)	0.013 (0.014)	0.307	0.492	0.010	1.117	0.430	0.565	0.539	0.704	0.786	0.274	0.949	0.105
Sorbose (439192)	0.014 (0.005)	0.021 (0.010)	0.014 (0.005)	0.013 (0.003)	0.099	0.680	0.123	0.902	0.034	0.900	0.996	0.002	0.969	0.032	1.000	0.034
Tagatose (439312)	0.091 (0.053)	0.163 (0.094)	0.088 (0.040)	0.086 (0.029)	0.043	1.187	0.034	0.984	0.004	1.876	0.952	0.053	0.786	0.364	0.949	0.488
Talose (441035)	0.149 (0.052)	0.252 (0.080)	0.143 (0.040)	0.130 (0.045)	0.002	1.318	0.001	1.848	0.001	2.307	0.595	0.468	0.972	0.022	0.949	0.355
Threonate (151152)	0.010 (0.004)	0.014 (0.005)	0.011 (0.004)	0.011 (0.005)	3.1x10 ⁻⁵	2.141	2.0x10 ⁻⁵	1.970	1.1x10 ⁻⁵	2.274	0.602	0.334	0.786	0.282	0.949	0.159
Uridine (6029)	0.001 (2.8x10 ⁻⁴)	0.001 (3.1x10 ⁻⁴)	4.5x10 ⁻⁴ (1.7x10 ⁻⁴)	0.001 (1.7x10 ⁻⁴)	0.883	0.135	0.039	0.942	0.833	0.092	0.008	1.659	0.106	0.890	0.949	0.235
Xylose (135191)	0.016 (0.008)	0.014 (0.008)	0.007 (0.004)	0.011 (0.007)	0.632	0.282	0.026	1.002	0.092	0.512	0.415	0.574	0.039	1.489	0.908	0.898

Lipid and associated intermediates																
α -Hydroxyoctanoate (94180)	8.1x10 ⁻⁴ (4.7x10 ⁻⁵)	1.2x10 ⁻⁴ (4.8x10 ⁻⁵)	7.5x10 ⁻⁵ (4.3x10 ⁻⁵)	8.0x10 ⁻⁵ (6.4x10 ⁻⁵)	0.005	0.908	0.036	0.943	0.127	0.827	0.854	0.139	0.842	0.132	1.000	0.004
α -Linolenate (5280934)	0.006 (0.002)	0.007 (0.003)	0.005 (0.002)	0.005 (0.002)	3.9x10 ⁻⁴	3.457	1.4x10 ⁻⁴	1.328	0.001	1.171	0.575	0.159	0.786	0.438	0.949	0.267
β -Hydroxyhexanoate (11829482)	3.2x10 ⁻⁴ (1.3x10 ⁻⁴)	0.001 (3.6x10 ⁻⁴)	3.6x10 ⁻⁴ (1.5x10 ⁻⁴)	3.0x10 ⁻⁴ (1.6x10 ⁻⁴)	1.2x10 ⁻⁵	4.231	1.4x10 ⁻⁴	1.820	2.6x10 ⁻⁵	2.058	0.540	0.449	0.786	0.309	0.949	0.204
5-Dodecenoate (5312377)	5.4x10 ⁻⁵ (1.1x10 ⁻⁴)	0.002 (0.001)	1.2x10 ⁻⁵ (1.5x10 ⁻⁴)	1.2x10 ⁻⁴ (1.5x10 ⁻⁴)	1.6x10 ⁻⁶	14.666	8.3x10 ⁻⁷	2.604	7.8x10 ⁻⁷	2.470	0.392	0.956	0.849	0.135	0.949	0.628
10-Heptadecenoate (5312434)	4.7x10 ⁻⁴ (4.1x10 ⁻⁴)	0.003 (0.001)	3.4x10 ⁻⁴ (3.0x10 ⁻⁴)	4.1x10 ⁻⁴ (3.8x10 ⁻⁴)	3.7x10 ⁻⁷	3.856	7.8x10 ⁻⁸	3.903	7.8x10 ⁻⁷	3.735	0.773	0.237	0.786	0.312	0.949	0.133
11,14-Eicosadienoate (5282805)	0.002 (0.001)	0.003 (0.001)	0.002 (0.001)	0.001 (0.001)	0.002	1.371	4.5x10 ⁻⁴	3.013	1.8x10 ⁻⁴	3.977	0.575	0.539	0.842	0.156	0.949	0.232
11-Eicosenoate (5282768)	0.001 (5.0x10 ⁻⁴)	0.003 (0.001)	0.001 (2.9x10 ⁻⁴)	0.001 (0.001)	1.1x10 ⁻⁵	3.222	1.9x10 ⁻⁵	1.745	0.001	1.706	0.958	0.117	0.786	0.193	0.949	0.261
Docosaehaenoate (445580)	0.008 (0.004)	0.009 (0.003)	0.006 (0.003)	0.007 (0.004)	0.160	0.422	0.004	1.000	0.106	0.633	0.514	0.341	0.508	0.371	0.949	0.080
Eicosapentaenoate (446284)	0.006 (0.004)	0.008 (0.003)	0.006 (0.002)	0.006 (0.003)	0.064	0.656	0.008	1.243	0.005	1.045	0.769	0.156	0.849	0.108	1.000	0.014
Glycerol (753)	0.084 (0.031)	0.492 (0.157)	0.083 (0.032)	0.092 (0.036)	9.8x10 ⁻⁹	5.342	5.0x10 ⁻⁹	4.412	3.7x10 ⁻⁸	4.219	0.741	0.232	0.926	0.051	0.949	0.185
Glycerol monopalmitate (3084463)	3.5x10 ⁻⁴ (1.2x10 ⁻⁴)	0.001 (3.8x10 ⁻⁴)	2.9x10 ⁻⁴ (7.5x10 ⁻⁵)	3.6x10 ⁻⁴ (2.2x10 ⁻⁴)	0.049	2.193	0.006	1.090	0.036	0.879	0.539	0.867	0.786	0.509	1.000	0.014
Heptadecanoate (10465)	0.003 (0.001)	0.006 (0.001)	0.003 (0.001)	0.003 (0.001)	5.9x10 ⁻⁵	2.805	3.0x10 ⁻⁶	2.626	7.3x10 ⁻⁵	2.628	0.996	0.002	0.786	0.242	0.949	0.244
Laurate (3893)	0.009 (0.003)	0.019 (0.005)	0.011 (0.006)	0.009 (0.003)	2.8x10 ⁻⁶	2.640	3.7x10 ⁻⁴	2.433	2.0x10 ⁻⁵	3.084	0.539	0.462	0.786	0.436	0.949	0.153
Methyl oleate (9922235)	9.9x10 ⁻⁵ (4.3x10 ⁻⁵)	1.7x10 ⁻⁴ (8.2x10 ⁻⁵)	8.2x10 ⁻⁵ (4.0x10 ⁻⁵)	1.2x10 ⁻⁴ (1.3x10 ⁻⁴)	0.035	1.593	0.015	1.070	0.274	0.606	0.575	0.931	0.786	0.491	0.949	0.413
Myristoleate (5281119)	4.8x10 ⁻⁴ (4.1x10 ⁻⁴)	0.006 (0.002)	3.4x10 ⁻⁴ (2.7x10 ⁻⁴)	4.5x10 ⁻⁴ (0.001)	2.1x10 ⁻⁸	6.616	5.0x10 ⁻⁹	4.497	3.7x10 ⁻⁸	4.360	0.769	0.284	0.786	0.364	0.949	0.151
Oleate (445639)	0.107 (0.042)	0.540 (0.200)	0.113 (0.036)	0.121 (0.066)	2.1x10 ⁻⁸	4.302	1.4x10 ⁻⁷	3.926	0.001	4.352	0.741	0.572	0.786	0.213	0.949	0.231
Palmitate (985)	0.398 (0.094)	0.665 (0.128)	0.368 (0.073)	0.381 (0.090)	6.7x10 ⁻⁶	2.153	4.2x10 ⁻⁷	3.410	2.1x10 ⁻⁵	3.261	0.773	0.131	0.786	0.274	0.949	0.168

Palmitoleate (445638)	0.007 (0.004)	0.041 (0.025)	0.008 (0.004)	0.009 (0.006)	0.035	1.711	0.036	0.761	0.084	0.679	0.741	0.206	0.786	0.309	0.949	0.460
Pentadecanoate (13849)	0.003 (0.001)	0.007 (0.003)	0.004 (0.002)	0.003 (0.001)	5.9x10 ⁻⁶	2.813	3.3x10 ⁻⁴	1.920	1.0x10 ⁻⁴	2.294	0.561	0.358	0.786	0.310	0.949	0.176
Ketones and TCA cycle intermediates																
α-Ketoglutarate (51)	0.002 (0.001)	0.003 (0.001)	0.002 (0.001)	0.002 (0.001)	0.046	1.267	0.003	1.060	0.011	0.723	0.595	0.323	0.786	0.282	0.949	0.088
β-Hydroxybutyrate (441)	0.018 (0.018)	0.232 (0.172)	0.013 (0.007)	0.014 (0.011)	1.7x10 ⁻⁶	3.621	1.5x10 ⁻⁷	2.854	4.8x10 ⁻⁷	2.843	0.987	0.022	0.786	0.230	0.949	0.214
Acetoacetate (96)	7.4x10 ⁻⁵ (5.9x10 ⁻⁵)	2.1x10 ⁻⁴ (1.9x10 ⁻⁴)	7.8x10 ⁻⁵ (5.1x10 ⁻⁵)	4.0x10 ⁻⁵ (5.6x10 ⁻⁵)	0.068	2.475	0.038	0.731	0.009	0.904	0.508	0.676	0.904	0.068	0.949	0.501
Citrate (311)	0.001 (3.6x10 ⁻⁴)	0.001 (0.001)	0.001 (3.9x10 ⁻⁴)	0.001 (3.9x10 ⁻⁴)	0.118	1.071	0.097	0.526	0.007	0.948	0.398	0.632	0.827	0.222	0.949	0.458
Fumarate (444972)	0.005 (0.002)	0.007 (0.003)	0.005 (0.002)	0.005 (0.002)	0.116	0.940	0.100	0.436	0.034	0.624	0.392	0.375	0.786	0.199	0.949	0.122
Malate (525)	0.004 (0.002)	0.007 (0.003)	0.004 (0.002)	0.003 (0.001)	0.008	1.284	0.003	1.146	0.001	1.294	0.705	0.187	0.849	0.183	0.949	0.372
Malonate (867)	3.1x10 ⁻⁴ (1.2x10 ⁻⁴)	4.3x10 ⁻⁴ (2.2x10 ⁻⁴)	3.1x10 ⁻⁴ (9.8x10 ⁻⁵)	2.7x10 ⁻⁴ (1.0x10 ⁻⁴)	0.191	0.906	0.157	0.605	0.039	0.859	0.569	0.476	0.972	0.016	0.949	0.403
AA and cholesterol-associated intermediates																
α-Ethylhydracrylate (188979)	0.002 (0.001)	0.006 (0.002)	0.003 (0.001)	0.003 (0.001)	7.1x10 ⁻⁵	2.284	2.0x10 ⁻⁴	1.464	2.1x10 ⁻⁴	1.513	0.887	0.058	0.842	0.143	1.000	0.071
α-Hydroxybutyrate (11266)	0.017 (0.010)	0.044 (0.024)	0.023 (0.015)	0.021 (0.017)	0.038	1.216	0.048	0.687	0.045	0.775	0.771	0.125	0.786	0.365	0.949	0.256
β-Hydroxyisobutyrate (87)	1.9x10 ⁻⁵ (3.1x10 ⁻⁵)	3.0x10 ⁻⁴ (1.4x10 ⁻⁴)	1.3x10 ⁻⁴ (2.4x10 ⁻⁵)	1.7x10 ⁻⁵ (2.6x10 ⁻⁵)	1.7x10 ⁻⁵	9.935	7.8x10 ⁻⁶	2.096	1.1x10 ⁻⁵	2.072	0.769	0.151	0.803	0.242	0.949	0.128
β-Hydroxyisovalerate (69362)	0.002 (0.001)	0.002 (0.001)	0.002 (0.001)	0.002 (0.001)	0.260	0.308	0.235	0.268	0.022	0.626	0.392	0.392	0.882	0.070	0.949	0.248
5-Pregnen-3β,20α-diol (312224064)	2.9x10 ⁻⁴ (2.8x10 ⁻⁴)	0.001 (4.1x10 ⁻⁴)	2.1x10 ⁻⁴ (2.1x10 ⁻⁴)	2.0x10 ⁻⁴ (1.6x10 ⁻⁴)	0.001	0.873	0.001	0.899	0.001	0.929	0.852	0.052	0.473	0.270	0.949	0.309
Alanine (5950)	3.7x10 ⁻⁴ (2.1x10 ⁻⁴)	2.4x10 ⁻⁴ (1.4x10 ⁻⁴)	0.001 (2.9x10 ⁻⁴)	4.4x10 ⁻⁴ (2.0x10 ⁻⁴)	0.125	0.615	0.004	2.108	0.001	1.416	0.539	0.394	0.473	0.820	0.949	0.349
Aminomalonate (100714)	0.051 (0.025)	0.024 (0.010)	0.051 (0.014)	0.050 (0.017)	0.075	1.186	0.009	1.131	0.009	1.085	0.771	0.127	0.799	0.186	0.949	0.129
Aspartate (5960)	0.021 (0.012)	0.013 (0.007)	0.026 (0.013)	0.017 (0.006)	0.254	0.587	0.004	0.898	0.129	0.539	0.392	0.579	0.786	0.348	1.000	0.026
Glutamate (33032)	0.005 (0.002)	0.004 (0.002)	0.005 (0.002)	0.005 (0.002)	0.580	0.429	0.039	0.726	0.074	0.536	0.640	0.363	0.786	0.404	0.949	0.187

Glutarate (743)	2.6x10 ⁻⁴ (2.1x10 ⁻⁴)	4.4x10 ⁻⁴ (3.2x10 ⁻⁴)	5.0x10 ⁻⁴ (4.5x10 ⁻⁴)	2.3x10 ⁻⁴ (1.7x10 ⁻⁴)	0.117	0.828	0.787	0.133	0.044	0.690	0.392	0.686	0.508	1.018	0.949	0.160
Glycine (750)	0.045 (0.020)	0.027 (0.014)	0.048 (0.018)	0.039 (0.016)	0.272	0.696	0.040	0.838	0.092	0.522	0.522	0.740	0.821	0.222	0.949	0.124
Hydroxyproline (5810)	0.003 (0.004)	0.002 (0.001)	0.003 (0.002)	0.004 (0.003)	0.233	0.545	0.067	1.130	0.003	1.609	0.643	0.415	0.893	0.093	0.949	0.364
Methionine (6137)	0.006 (0.004)	0.003 (0.002)	0.008 (0.003)	0.007 (0.004)	0.205	0.679	0.006	1.305	0.027	0.897	0.539	0.697	0.786	0.383	1.000	0.051
Phenylalanine (6140)	0.030 (0.016)	0.019 (0.010)	0.038 (0.009)	0.029 (0.012)	0.306	0.594	0.004	1.081	0.047	0.674	0.119	1.111	0.786	0.485	0.949	0.079
<i>p</i> -Hydroxyphenylacetate (127)	0.001 (0.001)	0.002 (0.002)	0.001 (0.001)	0.001 (0.001)	0.051	1.495	0.027	0.761	0.012	0.896	0.651	0.253	0.904	0.063	0.949	0.190
<i>p</i> -Hydroxyphenyllactate (9378)	0.003 (0.002)	0.005 (0.003)	0.003 (0.001)	0.003 (0.001)	0.084	0.834	0.037	0.627	0.007	0.794	0.656	0.236	0.893	0.064	0.949	0.141
Pyroglutamate (7405)	0.062 (0.013)	0.044 (0.011)	0.059 (0.015)	0.061 (0.018)	0.001	1.886	0.007	1.031	0.016	1.172	0.773	0.155	0.786	0.318	0.969	0.103
Squalene (638072)	0.001 (0.001)	0.004 (0.005)	0.001 (0.001)	0.001 (0.001)	0.027	1.700	0.039	0.818	0.043	0.803	0.980	0.039	0.795	0.176	0.949	0.203
Tyrosine (6057)	0.020 (0.008)	0.013 (0.006)	0.023 (0.007)	0.020 (0.007)	0.084	1.116	0.008	1.068	0.029	0.852	0.552	0.379	0.786	0.363	1.000	0.064
Valine (6287)	0.179 (0.096)	0.095 (0.065)	0.175 (0.104)	0.211 (0.134)	0.331	0.634	0.161	0.623	0.036	0.704	0.857	0.159	0.904	0.085	0.949	0.178

ANNEXURE B

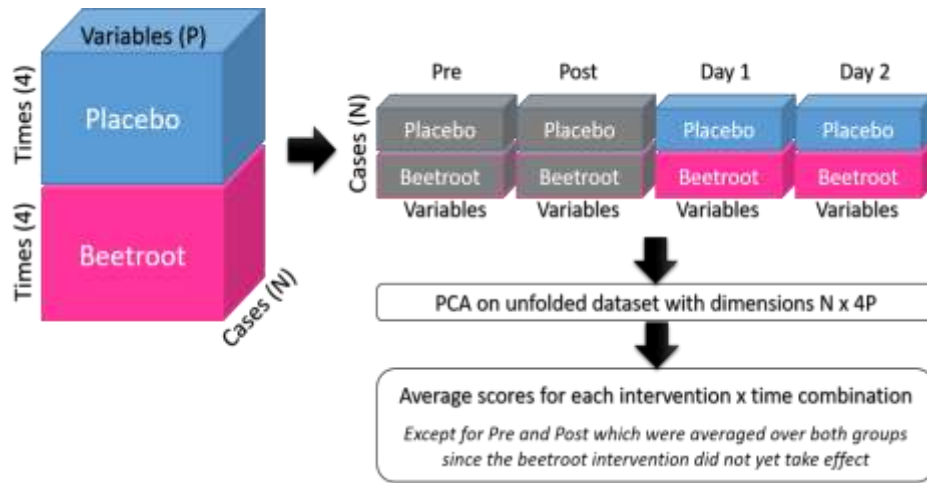


Figure B-1: A summary of the unfolded PCA of the beetroot and placebo interventions over time. Abbreviations: N: number of cases; P: number of variables.

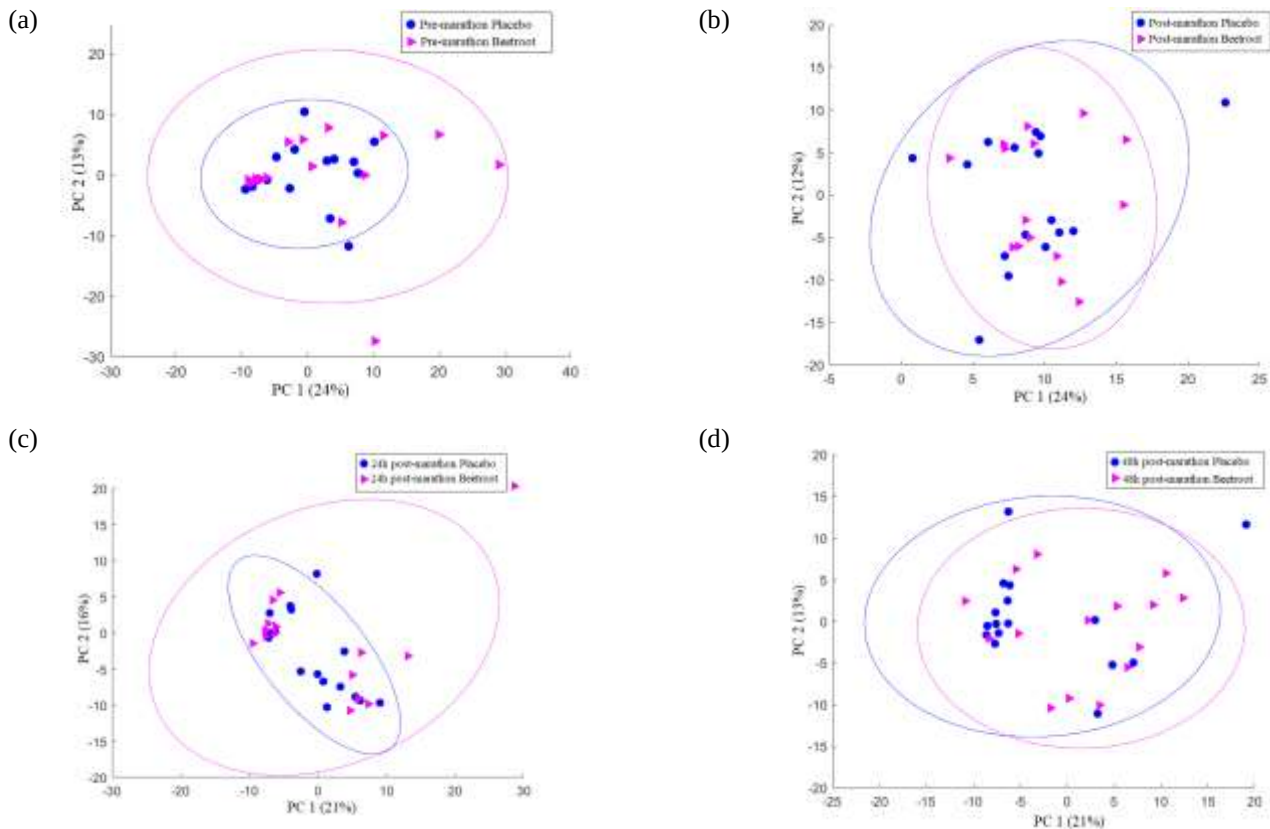


Figure B-2: Principal component analysis plots representing the inter-cohort differentiation of (a) pre-marathon, (b) post-marathon, (c) 24 h post-marathon, and 48 h post-marathon metabolic profiles. Abbreviations: PC: principal component.

Table B-1: Average concentrations and RM ANOVA *p*-values the metabolites selected to be significant pertaining to the aim of this investigation.

Metabolite (PubChem ID)	Group	RM ANOVA		Pre-marathon	Post-marathon	24 h post-marathon	48 h post-marathon
		Intervention p-value	Interaction p-value	Average concentration ±standard deviation			
α-Oleoylglycerol (5319879)	Placebo	0.42	0.01	0.001 ± 3.8×10 ⁻⁴	0.001 ± 3.6×10 ⁻⁴	4.3×10 ⁻⁴ ± 3.1×10 ⁻⁴	0.001 ± 3.9×10 ⁻⁴
	Beetroot			0.001 ± 0.001	4.1×10 ⁻⁴ ± 2.6×10 ⁻⁴	4.8×10 ⁻⁴ ± 3.2×10 ⁻⁴	0.001 ± 3.9×10 ⁻⁴
β-Hydroxydecanedioate (121232666)	Placebo	0.58	0.04	0.005 ± 0.001	0.006 ± 0.002	0.007 ± 0.002	0.005 ± 0.001
	Beetroot			0.006 ± 0.002	0.006 ± 0.002	0.006 ± 0.001	0.006 ± 0.003
β-Hydroxyphenylacetate (12122) ^a	Placebo	0.01	0.07	3.1×10 ⁻⁵ ± 4.6×10 ⁻⁵	2.8×10 ⁻⁵ ± 4.1×10 ⁻⁵	1.6×10 ⁻⁵ ± 3.1×10 ⁻⁵	2.0×10 ⁻⁵ ± 3.6×10 ⁻⁵
	Beetroot			4.3×10 ⁻⁵ ± 6.4×10 ⁻⁵	1.1×10 ⁻⁴ ± 1.3×10 ⁻⁴	3.2×10 ⁻⁵ ± 4.3×10 ⁻⁵	5.9×10 ⁻⁵ ± 4.3×10 ⁻⁵
β-Hydroxyvalerate (107802)	Placebo	0.02	0.72	5.3×10 ⁻⁴ ± 4.2×10 ⁻⁴	6.0×10 ⁻⁴ ± 3.9×10 ⁻⁴	6.8×10 ⁻⁴ ± 4.5×10 ⁻⁴	5.2×10 ⁻⁴ ± 1.7×10 ⁻⁴
	Beetroot			0.001 ± 0.001	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001
p-Hydroxyphenylacetate (127)	Placebo	0.02	0.92	0.001 ± 0.001	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001
	Beetroot			0.002 ± 0.001	0.003 ± 0.001	0.002 ± 0.001	0.002 ± 0.001
Arabitol (43925)	Placebo	2.9×10 ⁻⁴	1.4×10 ⁻⁸	0.004 ± 0.001	0.005 ± 0.002	0.004 ± 0.001	0.003 ± 0.001
	Beetroot			0.005 ± 0.002	0.005 ± 0.002	0.007 ± 0.002	0.007 ± 0.002
Eicosanoate (10467)	Placebo	0.01	0.95	4.2×10 ⁻⁴ ± 2.3×10 ⁻⁴	0.001 ± 4.1×10 ⁻⁴	4.6×10 ⁻⁴ ± 2.9×10 ⁻⁴	3.7×10 ⁻⁴ ± 2.0×10 ⁻⁴
	Beetroot			0.001 ± 4.4×10 ⁻⁴	0.001 ± 3.6×10 ⁻⁴	0.001 ± 0.001	0.001 ± 2.8×10 ⁻⁴
Glycerol (753)	Placebo	0.33	0.03	0.084 ± 0.031	0.492 ± 0.157	0.083 ± 0.032	0.092 ± 0.036
	Beetroot			0.092 ± 0.052	0.399 ± 0.114	0.091 ± 0.032	0.094 ± 0.047
Hippurate (464)	Placebo	0.02	0.73	0.002 ± 0.002	0.003 ± 0.004	0.003 ± 0.004	0.001 ± 0.002
	Beetroot			0.003 ± 0.003	0.005 ± 0.006	0.004 ± 0.004	0.004 ± 0.003
Mannitol (6251)	Placebo	0.01	0.06	0.003 ± 0.004	0.005 ± 0.004	0.004 ± 0.003	0.003 ± 0.003
	Beetroot			0.004 ± 0.004	0.01 ± 0.013	0.02 ± 0.03	0.008 ± 0.009
Nonanoate (8158)	Placebo	1.7×10 ⁻³	0.13	0.004 ± 0.002	0.004 ± 0.002	0.004 ± 0.001	0.003 ± 0.001
	Beetroot			0.005 ± 0.002	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001
Oxalate (971)	Placebo	0.04	0.24	0.013 ± 0.005	0.015 ± 0.006	0.014 ± 0.004	0.016 ± 0.007
	Beetroot			0.016 ± 0.006	0.02 ± 0.006	0.016 ± 0.005	0.015 ± 0.004
Rhamnose (25310)	Placebo	0.01	0.57	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
	Beetroot			0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
Threonate (21145021)	Placebo	0.07	0.02	0.01 ± 0.004	0.014 ± 0.005	0.011 ± 0.004	0.011 ± 0.005
	Beetroot			0.013 ± 0.005	0.018 ± 0.005	0.011 ± 0.002	0.013 ± 0.003
Undecanoate (8180)	Placebo	7.9×10 ⁻⁴	0.15	7.7×10 ⁻⁵ ± 5.7×10 ⁻⁵	1.3×10 ⁻⁴ ± 4.7×10 ⁻⁵	1.2×10 ⁻⁴ ± 6.2×10 ⁻⁵	9.1×10 ⁻⁵ ± 3.6×10 ⁻⁵
	Beetroot			1.5×10 ⁻⁴ ± 4.8×10 ⁻⁵	1.8×10 ⁻⁴ ± 3.9×10 ⁻⁵	1.4×10 ⁻⁴ ± 5.5×10 ⁻⁵	1.4×10 ⁻⁴ ± 3. ×10 ⁻⁵
Xylose (135191)	Placebo	0.01	0.02	0.016 ± 0.008	0.014 ± 0.008	0.007 ± 0.004	0.011 ± 0.007
	Beetroot			0.023 ± 0.013	0.012 ± 0.006	0.02 ± 0.012	0.017 ± 0.015

ANNEXURE C

The following is a list of all the publications emanating from this study (original proofs/publications succeeding list):

- Stander, Z., Luies, L. & Loots, D.T. 2020. The acute systematic biochemical adaptations induced by endurance running. Submitted for publication to *Biological Reviews* (Manuscript number: BRV-04-2020-0089). Impact factor: 10.28
- Stander, Z., Luies, L., Mienie, L.J., Keane, K.M., Howatson, G., Clifford, T., Stevenson, E.J. & Loots, D.T. 2018. The altered human serum metabolome induced by a marathon. *Metabolomics*, 14(150). Impact factor: 3.16
- Stander, Z., Luies, L., Mienie, L.J., Van Reenen, M., Howatson, G., Keane, K.M., Clifford, T., Stevenson, E.J. & Loots, D.T. 2020. The unaided recovery of marathon-induced serum metabolome alterations. *Scientific Reports*, 10(11060). Impact factor: 4.52
- Stander, Z., Luies, L., Van Reenen, M., Howatson, G., Keane, K.M., Clifford, T., Stevenson, E.J. & Loots, D.T. 2020. Beetroot juice – a suitable post-marathon metabolic recovery supplement? To be submitted to *International Journal of Behavioural Nutrition and Physical Activity*. Impact factor: 5.54



The acute systematic biochemical adaptations induced by endurance running

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Keywords:	Endurance running, Immunology, Metabolism, Physiology, Proficiency classification

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**The acute systematic biochemical adaptations induced by endurance
running**

Abbreviated title: Endurance running-induced biochemical adaptations

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Abstract

Endurance running started out as a health-promoting, recreational activity, but rapidly evolved into a highly competitive sport. Such strenuous endurance events are known to impact the human body on a physiological, immunological and metabolic level which may be classified as beneficial and/or detrimental. Although the effects of endurance activities have been thoroughly researched from a variety of perspectives, little has been done towards discussing the comprehensive systematic biochemical adaptations induced by endurance running. In this review, we comprehensively discuss the physiological, immunological, and metabolic-associated biomarker adaptations induced by endurance running while focusing on the factors that may influence these. Associations of these biological profiles with pathologies, internal damage and pathway complexity are also discussed, and a proficiency classification system for endurance runners is proposed. Furthermore, a comprehensive view of the effects of endurance running may provide clues to demographic-specific performance optimisation methods as well as the possible identification of more efficient systematic damage prevention strategies.

Keywords: Endurance running; immunology; metabolism; physiology; proficiency classification

Content

I.	Introduction.....	4
II.	Physiological (structural) and immunological biomarker adaptations	5
(1)	Blood biochemistry.....	5
(2)	Cardiovascular markers	20
(3)	Muscle, renal and hepatic damage markers	21
(4)	Immune response and inflammation.....	23
(5)	Structural damage and associated hormones	27
III.	Metabolomic biomarkers	30

1
2
3 25 (1) Carbohydrate metabolism and the tricarboxylic acid cycle.....41
4
5
6 26 (2) Lipid metabolism 43
7
8 27 (3) AA metabolism 45
9
10
11 28 (4) Purine metabolism, reactive oxygen species and vitamins.....48
12
13 29 IV. In a nutshell, what does this mean? 49
14
15 30 (1) The problem and its roots 49
16
17 31 (2) A possible proficiency classification system 50
18
19
20 32 V. Conclusion 52
21
22 33 VI. Additional information 53
23
24 34 (1) Author contribution.....53
25
26 35 (2) Funding statement and grant acknowledgements 53
27
28 36 (3) Conflict/declarations of interest.....53
29
30 37 (4) Compliance with ethical requirements 54
31
32
33
34 38 VII. References.....54
35
36 39 VIII. Appendix A.....70
37
38 (1) Abbreviations.....70
39
40
41
42
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44
45
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48
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I. Introduction

Endurance running (ER) is a highly competitive, well-established sport, that is categorised as either a long-distance race (>5 km) (Lieberman *et al.*, 2009), half-marathon (21.1 km), marathon (42 km) or ultra-marathon (>42 km) (Kruger & Saayman, 2013). Participation in these races has increased exponentially over the past decade, with participant rationales ranging from health-related benefits to intrinsic rewards and camaraderie (Kruger & Saayman, 2013). Running these distances require extensive mental and physical conditioning along with carefully planned personalised diets and extreme mental preparation. One of the most crucial factors adversely influencing athlete performance during an endurance run, is fatigue (Rapoport, 2010). Athlete performance may be influenced by a number of factors, including: (a) the depletion of fuel substrates, (b) the intensity and duration of muscle contractions, (c) maximum volume of oxygen (VO_{2max}) intake and (d) nutritional support during training/the race (Rapoport, 2010). Although many of these factors are controllable to some extent, numerous predictive factors are environmental (Oliveira *et al.*, 2017) and/or genetic (Grealy *et al.*, 2015) and are therefore difficult or impossible to control for example; forefoot bone length (Ueno *et al.*, 2018), ageing (Van Beek, Kirkwood & Bassingthwaite, 2016; Connick, Beckman & Tweedy, 2015), epigenetics (Ehlert, Simon & Moser, 2013), and a vast array of DNA polymorphisms that are determinants of individual endurance, cardiovascular fitness, mitochondrial function, psychosomatic skills and VO_{2max}, etc. (Ahmetov & Fedotovskaya, 2012; Znazen *et al.*, 2017; Sarzynski, Ghosh & Bouchard, 2017). Nevertheless, moderate physical activity is mainly considered beneficial to human health (Rowe, Safdar & Arany, 2014), especially with regards to body-weight regulation (Vaynman & Gomez-Pinilla, 2006; Venables & Jeukendrup, 2008), neurological stimulation and subsequent endorphin production (Dishman & O'Connor, 2009). On the other hand, ER has also been associated with various adverse effects, including severe mechanical shearing, tendon and nerve damage (Bonasia *et al.*, 2015), a compromised immunity, and the propensity to various acute/chronic pathologies. Cardiovascular dysfunction (Carbone *et al.*, 2017), pulmonary arterial hypertension (La Gerche, Roberts & Claessen, 2014), upper respiratory infection (Robson-Ansley *et al.*, 2012), as well as sudden

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3 68 cardiac arrest (Harmon *et al.*, 2016; Webner *et al.*, 2012), are some of the pathological risk-factors
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5 69 associated with ER.
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7 70 Besides these phenotypical changes, ER also has a profound biochemical effect on athletes. A
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9 71 comprehensive understanding of the latter provides clues to demographic-specific performance
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11 72 optimisation methods as well as improved systematic damage prevention strategies. Here, we discuss
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13 73 the previously identified biomarkers adaptations induced by ER (summarised in Tables 1 and 2) and
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15 74 discuss these in terms of their associated pathologies, internal damage and pathway complexity.
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17 75 Furthermore, since a standard means of classifying ER athletes according to experience/performance is
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19 76 currently lacking in literature, we suggest a proficiency classification system (Table 3).
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24 77 **II. Physiological (structural) and immunological biomarker adaptations**

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26 78 **(1) Blood biochemistry**

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28 79 The majority of the research investigating the biological effects of ER is based on the utilisation of
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30 80 blood samples (either the plasma and/or serum) as the preferred medium. Since blood serves as the
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32 81 primary transporting mechanism for all molecules excreted/produced in response to physiological and
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34 82 pathological stimuli (Psychogios *et al.*, 2011), it is considered a popular medium for immunological
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36 83 and metabolic investigations. Commonly measured blood parameters include platelets (PL),
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38 84 haematocrit (Hct), mean corpus haemoglobin concentration (MCHC), mean corpuscular volume
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40 85 (MCV), mean corpuscular haemoglobin volume (MCH), red blood cells (RBC, also called erythrocytes)
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42 86 and haemoglobin (Hb). Although the majority of the literature investigating the changes induced by
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44 87 ER, reported only minor and/or largely non-significant changes to these parameters after ER events
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46 88 (Table 1), a selected few observed significant fluctuations (Table 1). Kratz *et al.* (2006) investigated
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48 89 the effect of marathon running on various platelet-activation biomarkers in 32 healthy athletes
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50 90 participating in the Boston Marathon and observed elevated RBC, PL counts, Hct, Hb and MCHC.
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52 91 These elevations are supported by a few studies (Table 1) and are mainly ascribed to an acute-phase
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54 92 inflammatory response due to possible tissue damage, and/or dehydration (Kratz *et al.*, 2006; Widmaier,
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56 93 Raff & Strang, 2016). Although Da Ponte *et al.* (2018) also observed elevated PL and MCHC in 22
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58 94 Italian males participating in an uphill marathon, significant reductions in Hct were observed regardless
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of the mildly dehydrated state of the athletes. This coincides with the findings of Liu *et al.* (2018), in which a reduction in Hct was accompanied by reductions in RBC and elevations in haptoglobin, reticulocytes (immature RBC), and ferritin, in 19 male athletes participating in a 24 h ultra-marathon. Lui *et al.* (2018), attributed this observation to haemolysis that may be classified as ‘sports-anaemia’ (Chiu *et al.*, 2015; Wang *et al.*, 2010). The aforementioned haemolysis may be caused by a continuous mechanical force (foot-strike, rhabdomyolysis, etc.) shearing and the subsequent release of RBC content (Lippi & Sanchis-Gomar, 2019), and hence the reductions in the RBC, Hct, and haem (Robach *et al.*, 2014), accompanied by elevations of ferritin and iron. Elevated blood ferritin levels have also been associated with hepatic damage, poor iron status, and angiogenesis. Elevated levels of haptoglobin is crucial during haemolysis, as it is thought to react with Hb to prevent the oxidative capabilities of RBC components (Lippi & Sanchis-Gomar, 2019; Chiu *et al.*, 2015; Robach *et al.*, 2014). This is counteracted by the activation of erythropoiesis, leading to the elevations in the reticulocytes observed (Table 1).

Alternatively, a decrease in Hct has also been associated with haemodilution (Oliveira *et al.*, 2017) due to plasma volume expansion (Schumacher *et al.*, 2002; Dos Santos, 2018; Chlibkova *et al.*, 2018; Robach *et al.*, 2014) and/or overhydration (Hew-Butler *et al.*, 2017; Knechtle *et al.*, 2009) during and after prolonged endurance exercise. Knechtle *et al.* (2008) observed an increase in the total body water percentage in 10 Caucasian athletes after a 17 stage (1 200 km) ER event. Plasma volume expansion is likely subjective to (a) increased protein catabolism (Section III.3), since reduced skeletal muscle mass was also annotated in these athletes; (b) possible renal function impairment (Section II.3); and (c) ER-associated water and renal ion-regulating hormone (aldosterone and vasopressin) adaptations (Chlibkova *et al.*, 2018; Knechtle *et al.*, 2008). Elevated hormone production during and after ER events are regularly reported (Burge *et al.*, 2011; Siegel *et al.*, 2007; Niessner *et al.*, 2003) and are ascribed to the proposed initial system dehydration via excessive sweating/respiration (water and sodium $[Na^+]$ losses). The resulting reduction in plasma volume and increased plasma osmolality (Chlibkova *et al.*, 2018; Burge *et al.*, 2011; Knechtle *et al.*, 2008), may additionally result in the activation of the renin-angiotensin-aldosterone mechanism (Hew-Butler *et al.*, 2017; Widmaier *et al.*,

2016), and finally the promotion of fluid and Na⁺ re-absorption (plasma volume expansion). This mechanism serves as a preventive measure for a drop in blood pressure and fluid-electrolytes/hyponatremia (Hew-Butler *et al.*, 2017), as previously observed (Burge *et al.*, 2011; Knechtle, Knechtle & Rosemann, 2011; Kipps, Sharma & Tunstall Pedoe, 2011; Seal *et al.*, 2019; Goudie *et al.*, 2006). In accordance with the prevention of hyponatremia, Costa *et al.* (2013) investigated the hydration status and Na⁺ adaptations in 74 athletes after completing a five-stage ultra-marathon of 225 km. Despite the fact that the athletes ingested less Na⁺ (± 534 mg/L) than the suggested benchmark parameters (700–1 200 mg/L), the majority were still classified as normonatremic after the ER event, while only eight individuals (11%) presented with asymptomatic exercise-induced hyponatremia (Costa *et al.*, 2013). Seal *et al.* (2019) however, observed mild to severe hyponatremia in 41 of the 65 (63%) sampled athletes immediately after a 245 km ultra-marathon. It is worthy to note, however, that only 14 cases (34%) were associated with severe hyponatremia, while the remaining participants (n=27) developed only mild hyponatremia. As such, it could be suggested that hyponatremia may be dependent on more factors other than fluid intake and hydration status (Knechtle *et al.*, 2011), and factors such as the duration and intensity of the ER exercise bout.

Furthermore, Da Ponte *et al.* (2018), observed significantly elevated blood potassium (K⁺) and reduced chloride (Cl⁻) levels. Although these electrolyte changes (Table 1) have been ascribed to dehydration via excessive sweating (Mach & Fuster-Botella, 2017), elevated K⁺ has also been associated with rhabdomyolysis (Da Ponte *et al.*, 2018). Interestingly, elevated K⁺ after ER has been positively correlated with elevated aldosterone (Burge *et al.*, 2011), suggesting a possible association with the aforementioned renin-angiotensin-aldosterone mechanism's role in plasma volume expansion. Since these ions, along with calcium (Ca²⁺), are key components in processes associated with muscle contraction, such as the propagation of action potentials and excitation-contraction coupling (Hostrup & Bangsbo, 2017; MacLaren & Morton, 2012), perturbed homeostasis may result in impaired muscle contraction efficiency, force generation and an overall reduction in athletic performance. Factors determining the severity of the latter have been linked to age, as well as the intensity, duration and frequency of exercise (Hostrup & Bangsbo, 2017). Although all of these factors could contribute to the

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3 149 discrepancies in the blood parameter adaptations discussed in this section, it is hypothesised that the
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5 150 elapsed time between the cessation of ER and blood sampling could also lead to this variation since
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7 151 longer times allow for better hydration before post-exercise sampling (Kratz *et al.*, 2006).
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For Review Only

Table 1: Endurance running-induced physiological and immunological-associated biomarker adaptations reported in recent (2000-2020) literature (arranged according to publication year).

Reference	Exercise type	Cohort	Sampling media	Physiological and immunological adaptations when compared to baseline measurements	
Kratz <i>et al.</i> (2002)	Marathon	37 participants (32 male; 5 female)	Blood; before, within 4 h after and 24 h ⁺ post-race	Within 4 h post-race: Blood biochemistry: RBC (↓) ^a ; Hct (↓) ^a ; Hb (↑) ^a ; PL (↑) ^a ; Alb (↑) ^a ; TP (↑) ^a ; MCV (↓) ^a ; MCH (↑) ^a ; MCHC (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; ALT (↑) ^a ; AST (↑) ^a ; Bilirubin (↑) ^a ; ALP (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; cTnI (↑) ^a ; Mb (↑) ^a Renal damage: BUN (↑) ^a Electrolyte markers: Na ⁺ (↓)*; K ⁺ (↓)*; Cl ⁻ (↓) ^a ; Mg ²⁺ (↓) ^a ; Ca ²⁺ (↑) ^a ; P ³⁻ (↑) ^a Immune response: WBC (↑) ^a ; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Basophils (↑) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ; Globulin (↓) ^a	
Niessner <i>et al.</i> (2003)	Marathon	19 participants (17 male; 2 female)	Blood; before and immediately after the race	Post-race: Cardiac muscle damage: Pro-ANP (↑) ^a ; NT-proBNP (↑)* Hormonal response: Aldosterone (↑) ^a	
Uchakin, Gotovtseva & Stray-Gundersen (2003)	Marathon (White Rock and Cowton marathons)	15 male participants	Blood; before, 20 min and 60 min after the race as well as 2 ⁺ , 5 ⁺ and 8-days ⁺ post-race	Within 20 min post-race: Blood biochemistry: Hct (↑)*; Hb (↑)* Muscle and hepatic damage: CK (↑) ^a Immune response: Granulocytes (↑) ^a ; IL-6 (↑) ^a ; TNF-α (↑) ^a ; Lymphocytes (↓)*; IL-1β (-) LPS stimulated: IL-2 (↓) ^a ; IL-1β (↓)*; IFN-γ (↓) ^a ; IL-10 (↓)*; TNF-α (↓) ^a ; IL-6 (↓)* Hormonal response: ACTH (↑) ^a ; β-endorphin (↑) ^a ; Growth hormone (↑) ^a ; Cortisol (↑) ^a	Within 60 min post-race: Blood biochemistry: Hct (↑)* ^c ; Hb (↑)* ^c Muscle and hepatic damage: CK (↑) ^{a,b} Immune response: Granulocytes (↓) ^{a,c} ; IL-6 (↑) ^{a,c} ; TNF-α (↑) ^a ; Lymphocytes (↓) ^{a,c} ; IL-1β (-) LPS stimulated: IL-2 (↓) ^{a,c} ; IL-1β (↓)* ^b ; IFN-γ (↓) ^{a,c} ; IL-10 (↓)* ^b ; TNF-α (↓) ^{a,b} ; IL-6 (↓)* ^b Hormonal response: ACTH (↑) ^{a,c} ; β-endorphin (↑) ^{a,c} ; Growth hormone (↑) ^{a,c} ; Cortisol (↑) ^{a,c}
Goudie <i>et al.</i> (2006)	Marathon	14 participants (genders not provided)	Blood; patients admitted to St Thomas hospital post-race	Post-race: Electrolyte markers: Na ⁺ (↓) ^a	
Kratz <i>et al.</i> (2006)	Marathon	32 participants (27 male; 5 female)	Blood; before and immediately after the race	Post-race: Blood biochemistry: RBC (↓) ^a ; Hct (↑) ^a ; Hb (↑) ^a ; PL (↑)*; MCV (↓) ^a ; MCH (↑)*; MCHC (↑) ^a ; Reticulocyte (↑) ^a Immune response: WBC (↑) ^a ; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Basophils (↓) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ;	
Leers, Schepers & Baumgarten (2006)	Marathon	27 participants (25 male; 2 female)	Blood; before, immediately after and 24 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑) ^a Electrolyte markers: Na ⁺ (↓)*; Cl ⁻ (↓) ^a ; K ⁺ (-)	

Kim, Lee & Kim (2007)	Ultra-marathon (200 km)	54 male participants	Blood; before, after 100 km and immediately post-race	100 km of race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: Hs-CRP (↑) ^a ; IL-6 (↑) ^a ; TNF-α (↑)* Cartilage damage: COMP (↑) ^a Blood biochemistry: Hct (↑)*	Post-race: Muscle and hepatic damage: CK (↑) ^{a,d} ; LDH (↑) ^{a,d} ; AST (↑) ^{a,d} ; ALT (↑) ^a Immune response: Hs-CRP (↑) ^{a,d} ; IL-6 (↑) ^{a,d} ; TNF-α (↑)* Cartilage damage: COMP (↑) ^{a,d} Blood biochemistry: Hct (↑)* ^b
Mouzopoulos <i>et al.</i> (2007)	Ultra-marathon (245 km)	16 male participants	Blood; 5-days before, immediately after as well as 1 ⁺ , 3 ⁺ and 5-days ⁺ post-race	Post-race: Bone biochemistry: ICTP (↑)*; PICP (↓) ^a ; Osteocalcin (↓) ^a ; ALP (↓) ^a Hormonal response: Parathyroid hormone (↑) ^a Electrolyte markers: Ca ⁺ (↓)*	
Siegel <i>et al.</i> (2007)	Marathon	33 participants (<i>sex not provided</i>)	Blood; before and after the race	Post-race: Immune response: IL-6 (↑) ^a ; CRP (–) Muscle and hepatic damage: CK (↑) ^a Hormonal response: Prolactin (↑) ^a ; Arginine vasopressin (↑) ^a Renal damage: BUN (↑) ^a	
Lippi <i>et al.</i> (2008)	Half-marathon	15 male participants	Blood; before, immediately after, 3 h, ++	Post-race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; Mb (↑) ^a ; cTnT (–)	
Knechtle <i>et al.</i> (2009)	Ultra-marathon (100 km)	39 male participants	Blood and urine; before and immediately after	Post-race: Blood biochemistry: Hct (↓) ^a Electrolyte markers: Na ⁺ (↓) ^a	
Kersch-Schindl <i>et al.</i> (2009)	Ultra-marathon (246 km)	18 participants (16 male; 2 female)	Blood; before, within 15 min after as well as 3-days ⁺ post-race	Post-race: Bone biochemistry: CTX (↑) ^a ; Osteocalcin (↓) ^a ; Osteoprotegerin (↑) ^a ; RANKL (↑)*	
Fu <i>et al.</i> (2010)	Half-marathon	17 adolescent male participants	Blood; before, within 12 min after as well as 4 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a	
Burge <i>et al.</i> (2011)	Ultra-marathon (100 km)	50 male participants	Blood and urine; before and after the race	Post-race: Blood biochemistry: Hct (↓)*; Hb (↓)* Electrolyte markers: Na ⁺ (↑) ^a ; K ⁺ (↑) ^a Hormonal response: Aldosterone (↑) ^a ; Copeptin (↑) ^a	
Kipps <i>et al.</i> (2011)	Marathon	88 participants (53 male; 35 female)	Blood; before and after the race	Post-race: Electrolyte markers: Na ⁺ (↓) ^a	

Knechtle <i>et al.</i> (2011)	Ultra-marathon (100 km)	145 male participants	Blood; before and immediately after the race	Post-race Blood biochemistry: Hct (↓) ^a ; Hb (↓) ^a Electrolyte markers: Na ⁺ (↓)*		
Lippi <i>et al.</i> (2011)	Half-marathon	15 male participants	Blood; pre-fasting, 48 h after last training session, immediately after the race as well as 3 h ⁺ , 6 h ⁺ and 24 h ⁺ post-race	Post-race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; GGT (↑) ^a ; Bilirubin (↑) ^a ; ALT (–); ALP (–)		
McCullough <i>et al.</i> (2011)	Marathon	25 participants (12 male; 13 female)	Blood: before, after and 24 h ⁺ post-race	Within 4 h post-race: Blood biochemistry: Alb (↑) ^a ; TP (↑) ^a ; MCV (↓) ^a ; MCHC (↑) ^a ; MCH (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; ALT (↑) ^a ; AST (↑) ^a ; Bilirubin (↑) ^a ; ALP (↑) ^a ; Aldolase (↑) ^a , ALP (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; cTnI (↑) ^a ; BNP (↑) ^a Renal damage: BUN (↑) ^a ; Cystatin (↑)*; NGAL (↑) ^a ; KIM-1 (↑) ^a Electrolyte markers: Na ⁺ (↑)*; K ⁺ (↑) ^a Immune response: Globulin (–)		
La Gerche <i>et al.</i> (2012)	Marathon	9 cyclists ⁺ , 13 triathlon- ⁺ and 7 marathon participants	Blood; 2–3 weeks before, immediately after and 6–11 days ⁺ post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; BNP (↑) ^a (<i>correlated with cardio-graphic tests</i>) ⁺		
Waśkiewicz <i>et al.</i> (2012)	Ultra-marathon (±168.5 km; 24 h)	19 male participants	Blood; before, at marathon distance, within 12 h and immediately after the ultra-marathon	Marathon length: Blood biochemistry: RBC (↑)*; Hb (↑)*; Hct (↑)*; PL (↑)*; MCV (↓)*; CO ₂ (↓)*; O ₂ (↓)* Muscle and hepatic damage: CK (↑) ^a ; AST (–); ALT (–); GGT (–) Electrolyte markers: Na ⁺ (↑)*; K ⁺ (↑) ^a ; Ca ²⁺ (↓)* Immune response: WBC (↑) ^a ; Lymphocytes (↑)*; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Eosinophils (↓)*; Basophils (↑)*; IL-6 (↑) ^a , CRP (–);	12 h of running: Blood biochemistry: RBC (↑)* ^{a,c} ; Hb (↑)* ^{a,c} ; PL (↑)* ^{a,c} ; O ₂ (↑)* ^{a,b} ; Hct (↓)* ^{a,c} ; MCV (↓)* ^{a,c} ; CO ₂ (↓) ^{a,c} Muscle and hepatic damage: CK (↑) ^{a,b} ; AST (–); ALT (–); GGT (–) Electrolyte markers: Na ⁺ (↑)* ^b ; K ⁺ (↑) ^{a,b} ; Ca ²⁺ (↓) ^a Immune response: WBC (↑) ^{a,c} ; Lymphocytes (↓)* ^{a,c} ; Monocytes (↑) ^{a,b} ; Neutrophils (↑) ^{a,b} ; Eosinophils (↓) ^{a,c} ; Basophils (↑)* ^{a,c} ; IL-6 (↑) ^{a,b} , CRP (↑) ^a	Post-race: Blood biochemistry: RBC (↑)* ^{a,c} ; Hb (↑)* ^{a,c} ; PL (↑)* ^{a,c} ; O ₂ (↑) ^{a,b} ; Hct (↓)* ^{a,c} ; MCV (↓)* ^{a,c} ; CO ₂ (↓) ^{a,c} Muscle and hepatic damage: CK (↑) ^{a,b} ; AST (↑) ^a ; ALT (↑) ^a ; GGT (↑)* Electrolyte markers: Na ⁺ (↓)* ^{a,c} ; K ⁺ (↑) ^{a,b} ; Ca ²⁺ (↑)* ^b Immune response: WBC (↑) ^{a,b} ; Lymphocytes (↓)* ^b ; Monocytes (↑) ^{a,b} ; Neutrophils (↑) ^{a,c} ; Eosinophils (↓) ^{a,b} ; Basophils (↑) ^{a,-} ; IL-6 (↑) ^{a,b} , CRP (↑) ^{a,b}

Costa <i>et al.</i> (2013)	Multi-stage-marathon (225 km)	74 participants (46 male; 28 female) and 12 controls ⁺ (5 male; 7 female)	Blood; before and after each stage	Stages 1-5: Electrolyte markers: Na ⁺ (↓)* (<i>hyponatremia in eight athletes</i>)			
Klapcinska <i>et al.</i> (2013)	Ultra-marathon (48 h; 183–320 km)	7 male participants	Blood; before, after running 12 h, 24 h and within 10 min after the race as well as 24 h ⁺ and 28 h ⁺ post-race	12 h of running: Blood biochemistry: RBC (↑)*; Hct (–); Hb (↑)*; PL (↑) ^a ; MCV (↓) ^a ; MCH (↑)*; MCHC (↑) ^a ; pCO ₂ (↓) ^a Electrolyte markers: Na ⁺ (↓)*; K ⁺ (↑) ^a ; Ca ²⁺ (↓)* Iron panel: Ferritin (↑) ^a ; Iron (↓)* Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; Lymphocytes (↑)*	24 h of running: Blood biochemistry: RBC (–) ^c ; Hct (↓)* ^c ; Hb (↑)* ^c ; PL (↑) ^{a,c} ; MCV (↓) ^{a,c} ; MCH (↑)*; MCHC (↑) ^{a,b} ; pCO ₂ (↓) ^{a,b} Electrolyte markers: Na ⁺ (↓)* ^c ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) ^{a,c} Iron panel: Ferritin (↑) ^{a,b} ; Iron (↓)* ^b Immune response: WBC (↑) ^{a,c} ; Neutrophils (↑) ^{a,c} ; Monocytes (↑) ^{a,c} ; Lymphocytes (↑)*	Post-race: Blood biochemistry: RBC (↓)*; Hct (↓) ^a ; Hb (↑)*; PL (↑)* ^c ; MCV (↓) ^{a,b} ; MCH (↑)*; MCHC (↑) ^{a,c} ; pCO ₂ (↓) ^{a,c} Electrolyte markers: Na ⁺ (↓) ^{a,c} ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) ^{a,c} Iron panel: Ferritin (↑) ^{a,b} ; Iron (↓)* ^c Immune response: WBC (↑) ^{a,c} ; Neutrophils (↑) ^{a,c} ; Monocytes (↑) ^{a,c} ; Lymphocytes (↑)* ^c	
Robach <i>et al.</i> (2014)	Ultra-marathon (166 km)	22 male participants	Blood, before and within 20 min after the race as well as 2 ⁺ , 5 ⁺ , 9 ⁺ and 16 ⁺ –days post-race	Post-race: Blood biochemistry: Hb (↑)*, Haptoglobin (↓) ^a Cardiac muscle damage: CK-MB (↑) ^a ; Mb (↑) ^a ; cTnT (–) Muscle and hepatic damage: LDH (↑) ^a ; Bilirubin (↑)* Immune response: WBC (↑) ^a , CRP (↑) ^a , Orosomucoid (↑) ^a			
Salvagno <i>et al.</i> (2014)	Ultra-marathon (60 km)	18 participants (<i>sex not provided</i>)	Blood; before, within 10 min after as well as 1 h ⁺ after	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a ; Galectin-3 (↑) ^a			
Vuolteenaho <i>et al.</i> (2014)	Marathon	46 male participants	Blood; before and immediately after	Post-race: Cartilage damage: COMP (–); MMP (↑) ^a ; YKL (↑) ^a Hormonal response: Adiponectin (↑) ^a ; Leptin (↓)*; Resistin (↑) ^a			
Hewing <i>et al.</i> (2015)	Marathon	167 participants (78 male; 89 female)	Blood; before, immediately after as well as 2 weeks ⁺ post-race	Post-race: Blood biochemistry: Hb (↑) ^a ; Hct (↑) ^a ; TP (↑) ^a Electrolyte markers: Na ⁺ (↑) ^a Cardiac muscle damage: cTnT (↑) ^a ; NT-proBNP (↑) ^a Renal damage: Cystatin C (↑) ^a Immune response: CRP (↓) ^a			
Jastrzebski <i>et al.</i> (2015)-	Ultra-marathon (100 km)	14 male participants	Blood; before, after 25 km, 50 km, 75 km as well as	After 25 km: Muscle and hepatic damage: CK (↑) ^a , LDH	After 50 km: Muscle and hepatic damage: CK (↑) ^a , LDH	After 75 km: Muscle and hepatic damage: CK (↑) ^a , LDH	After 100 km: Muscle and hepatic damage: CK (↑) ^a , LDH

			immediately after the 100 km and 24 h ⁺ post-race	(↑) ^a , AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a	(↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a	(↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a	(↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a
Khodae et al. (2015)	Ultra-marathon (161 km)	20 pre-marathon participants (14 male; 6 female) and 21 post-marathon participants (17 male; 4 female)	Blood; before and immediately after	Post-race: Blood biochemistry: Hct (↓)*; Hb (↓)* Electrolyte markers: Na ⁺ (↓) ^a ; Cl ⁻ (↓) ^a ; K ⁺ (-) Renal damage: BUN (↑) ^a Cardiac muscle damage: cTnI (↑) ^a			
Kersch-Schindl et al. (2015)	Ultra-marathon (246 km)	19 participants (18 male; 1 female)	Blood; before, within 15 min after as well as 3-days ⁺ post-race	Post-race: Bone biochemistry: CTX (↑) ^a , Sclerostin (↓)*, Dkk-1 (↓) ^a , PINP (↓) ^a , Cathepsin K (↓)* Muscle and hepatic damage: Myostatin (↑) ^a , Follistatin (↑) ^a			
Li et al. (2015)	5 km race	18 participants (9 male; 9 female)	Saliva; before and 10 min after the race	Post-race: Salivary defence (sex dependent): IgA (↑) ^a ; α-amylase (↑) ^a , TP (↑) ^a , lactoferrin (↑)*			
Suzuki et al. (2015)	5 h running exercise (Participant A= 34.14 km; participant B=43.05 km)	2 female participants	Blood; before, within 60 min after as well as 1 ⁺ , 3 and 5 h post-race	Participant A post-race: Blood biochemistry: Alb (↓) Hormonal response: Insulin (↑); Glucagon (↑); ACTH (↑); Vasopressin (↑)		Participant B post-race: Blood biochemistry: Alb (↑) Hormonal response: Insulin (↓); Glucagon (↑); ACTH (↑); Vasopressin (↑)	
Arakawa et al. (2016)	Ultra-marathon (130 km)	18 middle-aged male participants	Blood; before, after 42 km (marathon), immediately after as well as 3 ⁺ , 5 ⁺ and 7 days ⁺ post-race	Marathon length: Blood biochemistry: RBC (↑)*; Hct (↑)*; Hb (↑)*; TP (↑) ^a ; Alb (↑) ^a ; MCV (↓)*; MCH (↑)*; MCHC (↑)* Muscle and hepatic damage: CK (↑)*; LDH (↑) ^a ; AST (↑)*; ALT (↑)*; GGT (↑)*; Bilirubin (↑)* Renal damage: BUN (↑)* Hormonal response: Insulin (↓) ^a ; Leptin (↑)*; Adiponectin (↑)* Immune response: WBC (↑) ^a ; IL-6 (↑) ^a ; CRP (-); TNF-α (↑)* Iron panel: Ferritin (↑)*		Post-race: Blood biochemistry: RBC (↑)* ^c ; Hct (↑)* ^c ; Hb (↑)* ^c ; TP (↑)* ^c ; Alb (↑) ^{a,c} ; MCHC (↑)* ^c ; MCV (↓) ^{a,c} ; MCH (↓)* ^c Muscle and hepatic damage: CK (↑) ^{a,b} ; LDH (↑) ^{a,b} ; AST (↑) ^{a,b} ; ALT (↑) ^{a,b} ; GGT (↑)* ^b ; Bilirubin (↑) ^{a,b} Renal damage: BUN (↑) ^{a,b} Hormonal response: Insulin (↓) ^{a,c} ; Leptin (↓) ^{a,c} ; Adiponectin (↑)* ^b Immune response: WBC (↑) ^{a,b} ; IL-6 (↑) ^{a,c,*} ; CRP (↑) ^{a,b} ; TNF-α (↓)* ^c Iron panel: Ferritin (↑) ^{a,b} ;	

Bekos <i>et al.</i> (2016)	Marathon and half-marathon	34 marathon participants (24 male; 10 female), 36 half-marathon participants (19 male; 17 female) and 30 sedentary controls ⁺ (17 male; 13 female)	Blood and urine; before and immediately after as well as 2–7 days ⁺ post-race	Half-marathon: Blood biochemistry: RBC (↑) ^a ; Hb (↑) ^a ; PL (↑) ^a ; MCV (↑) ^a ; MCH (↑) ^a ; MCHC (↑) ^a ; Hct (–) Muscle and hepatic damage: CK (–); GGT (–) Cardiac muscle damage: CK-MB (–); sST2 (–) Electrolyte markers: Serum Na ⁺ (↑) ^a Urine fraction Na ⁺ (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; RAGE (↑) ^a ; AGE-CML (↑) ^a ; Lymphocytes (↓) ^a ; Monocytes (↓) ^a ; CRP (–); esRAGE (–); IL-1RA (–); IL-33 (–) Apoptosis: Serum HMGB1 (↑) ^a ; Serum cytokeratin 18 (↑) ^a ; Urine cytokeratin 18 (↑) ^a		Marathon: Blood biochemistry: RBC (↑) ^a ; PL (↑) ^a ; MCV (↑) ^a ; MCH (↑) ^a ; MCHC (↑) ^a ; Hct (–); Hb (–) Muscle and hepatic damage: CK (↑) ^a ; GGT (–) Cardiac muscle damage: CK-MB (↑) ^a ; sST2 (↑) ^a Electrolyte markers: Serum Na ⁺ (↑) ^a Urine fraction Na ⁺ (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; AGE-CML (↑) ^{a,d} ; Lymphocytes (↓) ^a ; Monocytes (–); CRP (–); RAGE (–); esRAGE (–); IL-1 (–); IL-33 (–) Apoptosis: Serum HMGB1 (↑) ^a ; Serum cytokeratin 18 (↑); Urine cytokeratin 18 (↑) [*]	
Jastrzebski <i>et al.</i> (2016)	Ultra-marathon (100 km)	14 male participants	Blood; before, after 20 km, 50 km, 75 km, and immediately after and 24 h ⁺ post-race	25 km of race: Blood cells: Hct (↑) [*] ; Hb (↑) [*] ; PL (↑) [*] ; RBC (↓) [*] ; MCV (↓) [*] ; MCH (↓) [*] ; MCHC (↓) [*] Electrolytes: Na ⁺ (↓) [*] ; Ca ²⁺ (↓) [*] ; Cl [–] (↓) [*] ; K ⁺ (↑) [*] Cardiac muscle damage: Mb (↑) [*] Muscle and hepatic damage: Bilirubin (↑) [*] Immune response: WBC (↑) [*]	50 km of race: Blood cells: Hct (↑) [*] ; Hb (↑) [*] ; PL (↑) [*] ; MCHC (↑) [*] ; RBC (↓) [*] ; MCV (↓) [*] ; MCH (↓) [*] Electrolytes: Na ⁺ (↓) [*] ; Ca ²⁺ (↓) [*] ; Cl [–] (↑) [*] ; K ⁺ (↑) [*] Cardiac muscle damage: Mb (↑) ^a Muscle and hepatic damage: Bilirubin (↑) [*] Immune response: WBC (↑) [*]	75 km of race: Blood cells: Hct (↑) [*] ; Hb (↑) [*] ; PL (↑) [*] ; RBC (↓) [*] ; MCV (↓) [*] ; MCH (↓) [*] ; MCHC (↓) [*] Electrolytes: Na ⁺ (↓) [*] ; Ca ²⁺ (↓) [*] ; Cl [–] (↓) [*] ; K ⁺ (↑) [*] Cardiac muscle damage: Mb (↑) ^a Muscle and hepatic damage: Bilirubin (↑) [*] Immune response: WBC (↑)	Post-race: Blood cells: Hct (↑) [*] ; Hb (↑) [*] ; PL (↑) [*] ; MCHC (↑) [*] ; RBC (↓) [*] ; MCV (↓) [*] ; MCH (↓) [*] Electrolytes: Na ⁺ (↑) [*] ; K ⁺ (↑) [*] ; Ca ²⁺ (↓) [*] ; Cl [–] (↓) [*] Cardiac muscle damage: Mb (↑) ^a Muscle and hepatic damage: Bilirubin (↑) ^a Immune response: WBC (↑) [*]
Nielsen <i>et al.</i> (2016)	Marathon and half-marathon	14 male marathon participants, 16 half-marathon participants (8 male; 8 female) and 10 male ranger cadets ⁺	Blood; before and immediately after each race	Half-marathon length: Blood biochemistry: Hct (–); Hb (–); PL (↑) ^a Immune response: WBC (↑) ^a ; IL-1β (↑) [*] ; IL-6 (↑) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF-α (↓) [*] ; IL-2 (–); IL-4 (–); IL-5 (–); IL-12 (–); IFN-γ (–); GM-CFS (–); VEGF (–) LPS stimulated: IL-1β (↓) ^a ; IL-6 (↓) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF (↓) ^a		Marathon length: Blood biochemistry: Hct (–); Hb (–); PL (↑) ^a Immune response: WBC (↑); IL-6 (↑) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF-α (↑) [*] ; IL-1β (↓) [*] ; IL-4 (↓) [*] ; IL-12 (↓) [*] ; VEGF (↓) [*] ; IL-2 (–); IL-5 (–); IFN-γ (–); GM-CFS (–) No LPS stimulation of cytokines	
Shin <i>et al.</i> (2016)	Ultra-marathon (308 km)	17 marathon participants, 17 ultra-marathon (100 km)	Blood; before, at marathon distance, after 100 km and	Marathon distance: Blood biochemistry: TP (↑) ^a ; Alb (↑) ^a	After 100 km: Blood biochemistry: TP (↑) [*] ; Alb (↑) [*]	Post-race: Blood biochemistry: TP (↑) ^a ; Alb (↓) ^a	

		participants and 17 ultra-marathon participants (308 km) (<i>sex not provided</i>)	immediately post-race	Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑) [*] ; GGT (↑) ^a , ALP (↑) ^a Renal damage: BUN (↑) ^a	Muscle and hepatic damage: CK (↑) ^{a,d} ; LDH (↑) ^{a,d} ; AST (↑) ^{a,d} ; ALT (↑) ^{a,d} ; Bilirubin (↑) ^a ; GGT (↓) [*] , ALP (↑) [*] Renal damage: BUN (↑) ^{a,d}	Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑) ^a ; GGT (↑) ^{a,d} , ALP (↓) [*] Renal damage: BUN (↑) ^{a,d}
Danese <i>et al.</i> (2017)	Half-marathon	30 participants (21 male; 9 female)	Blood; before and immediately after the race	Post-race: Blood biochemistry: Hct (–); Hb (–)		
Mündermann <i>et al.</i> (2017)	Marathon	45 male participants	Blood; before, after a 10-week training period ⁺ , immediately after the marathon as well as 24 h ⁺ post-race	Post-race: Cartilage damage: COMP (↑) ^a Immune response: Hs-CRP (↓) ^a , TNF-α (↓) ^a , IL-6 (↑) ^a		
Oliveira <i>et al.</i> (2017)	Marathon (hot and temperate temperatures)	26 male participants	Blood and urine, before, immediately after as well as 1 ⁺ and 3-days ⁺ after	Post-race (hot temperature): Blood biochemistry: RBC (–); Hct (↓) ^a ; Hb (↑) [*] ; MCV (↓) ^a ; MCH (↑) ^a ; MCHC (↑) ^a , EPO (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) [*] ; GGT (↓) [*] ; Bilirubin (↑) [*] Immune response: WBC (↑) ^a ; Lymphocytes (↓) ^a , Monocytes (↑) ^a , Neutrophils (↑) ^a , Eosinophils (↑) [*] , Orosmuroid (↑) ^a , CRP (–) Iron panel: Ferritin (↑) ^a , Transferrin (↓) [*] , Iron (↑) [*] Cardiac muscle damage: cTnT (↑) ^a , proBNP (↑) ^a , Mb (↑) ^a Electrolytes: Na ⁺ (↑) ^a ; Mg ²⁺ (↓) ^a , K ⁺ (↑) ^a , Ca ²⁺ (–)	Post-race (temperate temperature): Blood biochemistry: RBC (↓) ^a ; Hct (↓) ^a ; Hb (↓) [*] ; MCV (–); MCH (↑) ^a ; MCHC (↑) ^a , EPO (↑) [*] Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; GGT (↑) ^a ; Bilirubin (↑) ^a Immune response: WBC (↑) ^a ; Lymphocytes (↓) ^a , Monocytes (↑) ^a , Neutrophils (↑) ^a , Eosinophils (↓) ^a , Orosmuroid (↑) ^a , CRP (↓) ^a Iron panel: Ferritin (↑) ^a , Transferrin (↑) ^a , Iron (↑) [*] Cardiac muscle damage: cTnT (↑) ^a , proBNP (↑) ^a , Mb (↑) ^a Electrolytes: Na ⁺ (↑) [*] ; Mg ²⁺ (↓) ^a , K ⁺ (↓) [*] , Ca ²⁺ (↑) ^a	
Sansoni <i>et al.</i> (2017)	Ultra-marathon (65 km)	17 male participants and 12 age matched, moderately active controls	Blood; before and immediately post-race	Post-race: Bone biochemistry: P1NP (↓) ^a ; Ga-OC (↑) [*] ; Glu-OC (↓) ^a Hormonal response: Glucagon (↑) ^a ; GLP-1 (↑) ^a ; Resistin (↑) ^a ; Visfatin (↑) ^a ; Ghrelin (↑) [*] ; GIP (↑) [*] ;Leptin (↓) ^a , C-peptide (↓) ^a ; Insulin (↓) [*] .		
Zakovska <i>et al.</i> (2017)	Ultra-marathon (100 km; freezing conditions)	15 participants (12 male; 3 female)	Blood; before and immediately post-race	Post-race: Blood cells: RBC (↓) [*] ; Hct (↓) [*] ; Hb (↓) [*] ; PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; ALT (↓) [*]		

				Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; IgA (↑)*; IgM (↑)*; IgG (↑) ^a ; Lymphocytes (↓)*; Eosinophils (↓)*		
Chlibkova <i>et al.</i> (2018)	Multistage ultra-marathon (n=7 consecutive marathon races)	26 participants (20 male; 6 female)	Blood and urine; before and after stages 1, 4 and 7	After stage 1: Blood biochemistry: Hct (↓) ^a Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (–), Urine: K ⁺ (↑) ^a ; Na ⁺ (↓) ^a	After stage 4: Blood biochemistry: Hct (↓) ^{a,c} Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (↑)* ^d , Urine: K ⁺ (↑) ^{a,d} ; Na ⁺ (↓)*	After stage 7: Blood biochemistry: Hct (↓) ^a Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (↓)*, Urine: K ⁺ (↑) ^{a,c} ; Na ⁺ (–)
Da Ponte <i>et al.</i> (2018)	Marathon (up-hill only)	22 male participants	Blood; before and immediately post-race	Post-race: Blood biochemistry: RBC (–); Hb (–); Hct (↓) ^a ; MCV (↓) ^a ; MCH (↓) ^a ; PL (↑) ^a ; TP (↑) ^a ; Alb (↑) ^a ; MCHC (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a Cardiac muscle damage: cTnI (↑) ^a ; Mb (↑) ^a Electrolytes: K ⁺ (↑) ^a ; Na ⁺ (↓)*; Cl [–] (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; CRP (↑) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ; Basophil (–)		
Li <i>et al.</i> (2018)	Ultra-marathon (400 km)	16 male participants	Blood; before and immediately after the race	Post-race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a		
Liu <i>et al.</i> (2018)	Ultra-marathon (±153.64 km; 24 h)	19 male participants	Blood; before and immediately after	Post-race: Blood biochemistry: Reticulocyte (↑) ^a ; RBC (↓) ^a ; Hct (↓) ^a ; Hb (↓) ^a ; Haptoglobin (↓) ^a ; MCV (↓)*; MCH (↓) ^a ; MCHC (–) Iron panel: Ferritin (↑) ^a ; Iron (↑)*		
Park <i>et al.</i> (2018)	Ultra-marathon (100 km)	22 male participants (11 with EIH and 11 with normal exercise blood pressure)	Blood; before, immediately after, 24 h ⁺ , 72 h ⁺ and 120 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: CRP (↑) ^a		
Shin, Park & Kim (2018)	Ultra-marathon (622 km)	10 middle-age male participants	Blood; before, at 300 km, immediately after, as well as 3 ⁺ and 6-days ⁺ post-race	300 km of running: Blood biochemistry: RBC (↓) ^a , PL (↓)*, Hct (↓)*; Hb (↓) ^a Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: Hs-CRP (↑) ^a , WBC (↑) ^a	Post-race: Blood biochemistry: RBC (↓) ^a , PL (↑)*, Hct (↓)*; Hb (↓) ^a Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: Hs-CRP (↑) ^a , WBC (↑) ^a	
Tirabassi, Olewinski & Khodaei (2018)	Ultra-marathon (100 km)	36 participants (28 male; 8 female)	Blood; before and within 30 min after the race	Post-race: Blood biochemistry: TP (↓) ^a , Alb (↓) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑) ^a		

				Immune response: ALP (↓)*		
Aengevaeren <i>et al.</i> (2019)	Marathon	82 participants (65 male; 17 female)	Blood; before and immediately after the race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; sST2 (↑) ^a		
Bekos <i>et al.</i> (2019)	Marathon	34 marathon participants (24 male; 10 female); 36 half-marathon participants (19 male; 17 female); 30 sedentary volunteers ⁺ (17 male; 13 female)	Blood; before, immediately after as well as 2–7 days ⁺ after the marathon	Half-marathon athletes: Immune response: HSP70 (↑) ^a ; HSP27 (↑) ^a , WBC (↑); PL (↑) – <i>In individuals showing signs of EIB</i>	Marathon athletes: Immune response: HSP70 (↑) ^a ; HSP27 (↑) ^a ; WBC (↑); PL (↑) – <i>In individuals showing signs of EIB</i>	
Górecka <i>et al.</i> (2019)	Ultra-marathon	10 male participants	Blood; before, immediately after as well as 90 min post-race ⁺	Post-race: Blood biochemistry: Angiopoietin-like protein 4 (↑) ^a		
Hoppel <i>et al.</i> (2019)	Ultra-marathon (67 km)	8 male participants	Blood; before, after as well as 24 h ⁺ post-race	Post-race: Blood biochemistry: RBC (↑)*, Hct (↑)*; PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Renal damage: BUN (↑) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; CRP (↑) ^a ; Lymphocytes (↓)*; Eosinophils (↓)* Electrolyte markers: Na ⁺ (↓)*; K ⁺ (↑)*; Cl ⁻ (↓)*; Mg ²⁺ (↑)*; Ca ²⁺ (↑) ^a ;		
Le Goff <i>et al.</i> (2019)	Marathon, half-marathon and 1 h duration exercise	23 male marathon participants, 15 male half-marathon participants and male 17 untrained runners ⁺	Blood; before and immediately after each exercise as well as 3 h ⁺ post-race	1 h running: Blood cells: Hb (–) Muscle and hepatic damage: CK (↑) Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑)*; Galactin-3 (↑) ^a ; CK-MB (↑)*; Mb (↑)* Immune response: CRP (↑)*; Myeloperoxidase (↑)* Renal damage: Cystatin (↑)*	Half-marathon: Blood cells: Hb (↑)* Muscle and hepatic damage: CK (↑) Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑) ^a ; Galactin-3 (↑) ^a ; CK-MB (↑)*; Mb (↑)* Immune response: CRP (↑)*; Myeloperoxidase (↑)* Renal damage: Cystatin (↑)*	Post-race: Blood cells: Hb (↑)* Muscle and hepatic damage: CK (↑) Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑) ^a ; Galactin-3 (↑) ^a ; CK-MB (↑)*; Mb (↑)* Immune response: CRP (↑)*; Myeloperoxidase (↑)* Renal damage: Cystatin (↑)*
Martin <i>et al.</i> (2019)	Marathon	22 participants (9 male; 13 female)	Blood; 24 h before, immediately after	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; BNP (↑) ^a		

			as well as 24 h ⁺ post-race ⁺			
Rubio-Arias <i>et al.</i> (2019)	Ultra-marathons (54 km and 11 km)	16 male athletes (54 km n=10; 111 km n=6)	Blood; before and immediately after, as well as 24 h ⁺ , 48 h ⁺ and 72 h ⁺ post-race	After 54 km ultra-marathon: Blood biochemistry: RBC (↑)*; Hb (↑)*; Hct (↑)*; MCV (↑)*; MCH (–); PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a , ALT (↑) ^a , Mb (↑) ^a Cardiac muscle damage: cTnI (↑) ^a Immune response: WBC (↑) ^a , CRP (↓)*	After 111 km ultra-marathon: Blood biochemistry: RBC (↑)*; Hb (↓)*; Hct (↓)*; MCV (↓)*; MCH (↓)*, PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST/GOT (↑) ^a , ALT/GPT (↑) ^a , Mb (↑) ^a Cardiac muscle damage: cTnI (↑)* Immune response: WBC (↑) ^a , CRP (↓) ^a	
Seal <i>et al.</i> (2019)	Ultra-marathon (246 km)	63 male participants	Blood: before, at 93 km and within 15 min after	93 km of running: Electrolytes: Na ⁺ (↓) ^a	Post-race: Electrolytes: Na ⁺ (↓) ^a	
Sierra <i>et al.</i> (2019)	Marathon	31 male participants	Sputum (induced); before, immediately after as well as 24 h ⁺ and 72 h ⁺ post-race	Airway immune response; post-race: Neutrophils (↑) ^a ; Macrophages (↑) ^a ; Epithelial cells (↑) ^a ; Lymphocytes (↓)*; IL-6 (↓)*; IL-8 (↓)*; IL-23 (↓) ^a ; IL-33 (↓) ^a ; IL-12p40 (↓) ^a ; TSLP (↓) ^a		
Zebrowska <i>et al.</i> (2019)	Ultra-marathon (24 h; 149 km ± 33 km)	14 male participants	Blood; before, after marathon length as well as 12 h, and 48 h post-race ⁺	Marathon length: Cardiac muscle damage: NT-proBNP (↑) ^a ; CK-MB (↑)*; cTnT (↑)*; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑)*	12 h of running: Cardiovascular damage: NT-proBNP (↑)* ^c ; CK-MB (↑)* ^b ; cTnT (↑)* ^b ; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑) ^{a,d}	Post-race: Cardiovascular damage: NT-proBNP (↑)* ^c ; CK-MB (↑) ^{a,d} ; cTnT (↑) ^{a,d} ; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑) ^{a,d}
Malek <i>et al.</i> (2020)	Ultra-marathon (100 km)	18 participants (15 male; 3 female)	Blood; before and immediately post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a Immune response: Hs-CRP (↑) ^a		

Footnote: Biochemical changes are denoted as elevated (↑) or reduced (↓), with significance of these changes indicated as follows: significant (a) and non-significant (*) compared to baseline; non-significant/unreported elevations compared to preceding time point (b); non-significant/unreported reduction compared to preceding time point (c); significantly elevated compared to preceding time point (d), significantly reduced compared to preceding time point (e), unchanged concentration (–), data not included in table (+). **Abbreviations:** ACTH: adrenocorticotrophic hormone; AGE: advance glycation end-products; Alb: albumin; ALP: alkaline phosphatase; ALT: alanine transferase; AST: aspartate transferase; BNP: brain natriuretic peptide; BUN: blood-urea-nitrogen; Ca²⁺: calcium; CK: creatine kinase; CK-MB: creatine kinase-myocardial band; Cl[–]: chloride; CO₂: carbon dioxide; COMP: cartilage oligomeric matrix protein; CRP: C-reactive protein; cTnI: cardiac

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3 troponin I; cTnT: cardiac troponin T; CXT: cross-linked C-telopeptide of type I collagen; Dkk-1: dickkopf-1; EIH: exercise-induced hypertension; EPO: erythropoietin; esRAGE: endogenous
4 secretory RAGE; Ga-OC: carboxylate osteocalcin; GGT: gamma-glutamyl transferase; GIP: gastric inhibitory polypeptide; GLP-1: glucagon-like peptide; Glu-OC: undercarboxylated osteocalcin;
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6 GM-CFS: granulocyte-macrophage colony-stimulating factor; Hb: haemoglobin; Hct: haematocrit; H-FABP: heart-type fatty acid-binding protein; HMGB1: high mobility group box 1; Hs-CRP:
7 heat sensitive CRP; HSP: heat shock protein; ICTP: cross-linked carboxy-terminal telopeptide of type I collagen; IFN- γ : interferon-gamma; Ig: immunoglobulin; IL: interleukin; IMA: ischemia-
8 modified albumin; K⁺: potassium; KIM-1: kidney injury molecule-1; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; Mb: myoglobin; MCH: mean corpuscular haemoglobin volume; MCHC:
9 mean corpus haemoglobin concentration; MCV: mean corpus volume; Mg²⁺: magnesium; Na⁺: sodium; NGAL: neutrophil gelatinase-associated lipocalin; NT-proBNP: N-terminal pro-BNP; O₂:
10 oxygen; PINP: pro-collagen type I N-terminal pro-peptide; P³⁻: phosphate; PICP: procollagen I carboxyterminal pro-peptide; PL: platelets; Pro-ANP: pro-atrial natriuretic peptide; RAGE:
11 receptor of advance glycation end-products; RANKL: receptor activator of nuclear factor κ B ligand; RBC: red blood cell; sST2: suppression of tumorigenicity 2; TNF- α : tumour necrosis factor
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13 alpha; TP: total protein; TSLP: thymic stromal lymphopoietin; VEGF: vascular endothelial growth factor; WBC: white blood cells/leukocytes.
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(2) Cardiovascular markers

Although moderate-intensity exercise is eminent for its beneficial effects on cardiovascular functionality and cardiorespiratory fitness, the degree of exercise intensity that accompanies ER appears to have deleterious effects on these systems (Zebrowska *et al.*, 2019). Cardiovascular infarction or dysfunction and related pathologies are diagnosed using a wide variety of analytical techniques, of which biochemical fluctuations of cardiac troponin T (cTnT) and I (cTnI) are considered the gold standard (Park *et al.*, 2017; Scharhag *et al.*, 2008). Additional biomarkers used for verifying cardiac complications/damage include elevated levels of myoglobin (Mb) and creatine kinase-myocardial band (CK-MB) (Scharhag *et al.*, 2008; Kim *et al.*, 2015), and are generally observed in ER athletes (Table 1). However, since CK-MB is not specific to cardiac distress, and has also been associated with skeletal muscle damage (Donnellan & Phelan, 2018), elevations in this marker alone cannot attest to cardiovascular dysfunction in an ER cohort. Park *et al.* (2018) investigated the changes in various cardiac and muscle damage biomarkers in 11 athletes with exercise-induced hypertension (EIH). The results not only supported the elevations in CK-MB and cTnI, but also indicated elevated N-terminal pro-brain natriuretic peptide (NT-proBNP) concentrations. Natriuretic peptides are secreted by the heart in response to volume and wall stretching (Daniels & Maisel, 2007), and have been implicated to be a marker for cardiovascular dysfunction (Di Castelnuovo *et al.*, 2019). Nevertheless, Park *et al.* (2018) ascribed the aforementioned elevated NT-proBNP in athletes with EIH to increased myocardial workload and increased volume pressure associated with EIH, rather than actual cardiovascular dysfunction. Le Goff *et al.* (2019) also indicated elevated brain natriuretic peptide (BNP), in conjunction with increased galectin-3, when comparing the cardiovascular biomarker dynamics of untrained, half-marathon and marathon male athletes immediately post-ER. Similarly to BNP, galectin-3 proteins are secreted in response to myocardiocyte stretching, necrosis and cardiac fibrosis (Salvagno *et al.*, 2014; Le Goff *et al.*, 2019; Cheng *et al.*, 2013). Interestingly, Martin *et al.* (2019) observed significantly higher BNP concentrations in female athletes compared to their male counterparts. This suggests increased susceptibility of female athletes to cardiac stress, however, since no correlation could be made between gender and cTnI (Martin *et al.*, 2019), further research is required to establish if

females may possess a predisposition pertaining to exercise-induced cardiac stress. Similarly, Leers *et al.* (2006) also detected increased cTnT, BNP and NT-proBNP in athletes (n=27) participating in the Visé-Maastricht-Visé marathon. These increases were however transient, reflecting only temporary myocardial stunning, rather than prolonged damage, and were positively correlated with age, therefore suggesting that these markers may be simultaneously dependent on more than one factor.

Additionally, Zebrowska *et al.* (2019) investigated the acute effects of a 24 h ultra-marathon on novel cardiac markers, e.g. heart-type fatty acid-binding protein (H-FABP) and ischemia-modified albumin (IMA), in order to determine the functionality of these as possible early detection cardiovascular dysfunction biomarkers. Severe elevations in NT-BNP and H-FABP during the 24 h ultra-marathon (particularly at marathon length) supported the phenomenon of myocardial overload and hypoxia-induced myocardial damage and/or increased sarcolemma permeability (Zebrowska *et al.*, 2019), correlating with previous findings (Table 1). However, no significant (statistical) difference was observed in the IMA concentrations when compared to baseline values, which was attributed to myocardial protection and the possible increase in anti-oxidant capacity of athletes who exercise/compete on a regular basis (Zebrowska *et al.*, 2019). Aengevaeren *et al.* (2019) further support the structural toll of ER on cardiac muscle by reporting a significant suppression of tumorigenicity 2, particularly in faster athletes, immediately after a marathon. Although suppression of tumorigenicity 2 is predictive of ventricular overload and myocardial fibrosis, this biomarker is also associated with inflammation (Aimo *et al.*, 2019) and may in this case also indicate elevated inflammatory responses due to exercise-induced rhabdomyolysis.

(3) Muscle, renal and hepatic damage markers

Considering that ER involves a combination of concentric and eccentric muscle contractions, it has been suggested ER inflicts more muscle damage in comparison with other endurance exercises, such as cycling and cross-country skiing (De Oliveira Assumpção *et al.*, 2013). Mechanically, repeated high-forced eccentric contractions in ER primarily disrupt muscle ultra-structure, leading to a cascade of events associated with Ca^{2+} efflux, degradation of structural proteins, and membrane shearing, subsequently releasing numerous muscle-damage associated proteins into the circulatory system

(Owens *et al.*, 2019). The aforementioned ultimately leads to cellular apoptosis and/or necrosis, which results in the activation of inflammatory signalling mechanisms to remove cellular debris and initiate repair (Owens *et al.*, 2019; Baird *et al.*, 2012). Lippi *et al.* (2008) investigated the acute biochemical adaptations in 15 Caucasian male athletes after completing a half-marathon and observed significantly elevated creatine kinase and Mb in the post-race samples comparatively, which was attributed to increased membrane permeability due to muscular damage induced by the race. Robach *et al.* (2014) also support the notion of muscle damage as a result of ER and additionally showed elevated concentrations of orosomucoid in 22 male athletes after a 166 km ultra-marathon. This marker plays a role in the acute phase immune response and is known as the anti-fatigue protein that enhances muscle endurance during strenuous activity (Sun *et al.*, 2018; Robach *et al.*, 2014). Animal studies have shown, that orosomucoid is suppressed by oestrogen via p38 mitogen-activated protein kinase in female rats (Sun *et al.*, 2018), thus suggesting that sex may be a determinant factor of athlete muscle performance. Shin *et al.* (2016) investigated biochemical marker fluctuations during three different endurance races (i.e. marathon, 100 km and 308 km) in 15 demographically similar athletes. Elevated lactate dehydrogenase, aldolase, and blood-urea-nitrogen (BUN) were observed and ascribed to muscle and subsequently renal damage (rhabdomyolysis) (Shin *et al.*, 2016; Hoppel *et al.*, 2019). Both rhabdomyolysis and protein catabolism results in nitrogen release, and consequently the production of urea in the liver (Garrett & Grisham, 2013). Since urea build-up is detrimental to human health, this waste product is removed via the renal system. As such, elevated BUN may be indicative of hepatic and/or renal dysfunction. In accordance with the latter, McCullough *et al.* (2011) observed elevated levels of cystatin C, neutrophil gelatinase-associated lipocalin, kidney injury molecule-1 and serum creatinine (discussed in Section III.3) in 25 athletes who participated in the Flagstar Marathon. These markers are also strongly associated with acute renal injury (Murty *et al.*, 2013; Bonventre, 2014; Khawaja *et al.*, 2019), and elevations in any of these may depict transient renal injury or failure after ER, depending on the exercise severity, which concurs with numerous other investigations (Table 1). Furthermore, Rubio-Arias *et al.* (2019), similarly investigated the biochemical adaptations in 16 athletes running 54 km (n=10) or 111 km (n=6), and observed substantially elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST) concentrations, especially after running 111 km.

Although these markers may be indicative of muscle damage, the ratio of ALT to AST (elevated ALT) supports the phenomenon of liver damage, considering ALT is only found in small quantities in muscle cells. These transferases enzymes may also be elevated as both are key components in the metabolic attempt to replenish glucose stores via gluconeogenesis (Section III.3). Considering that these elevations progressively increase concurrently with the distance covered, it may suggest that distance is a depending factor of both muscle and liver damage. This observation was supported by the study of Jastrzebski *et al.* (2015) in which ALT and AST were elevated in 14 athletes after running 25 km, 50 km, 75 km and 100 km. However, these fluctuations were only statistically significant in older athletes at distances >50 km and could be ascribed to the possibility of older subjects possessing reduced hepatic adaptive abilities compared to younger athletes (Jastrzebski *et al.*, 2015). Contradictory to this, however, Tirabassi *et al.* (2018) did not observe any correlation between the elevations of ALT/AST detected and the age, sex, or the rate of distance covered by the athletes (n=36) after a 161 km ultra-marathon. In addition, Shin *et al.* (2016) investigated the adaptations of other hepatic damage-associated markers such as alkaline phosphatase, gamma-glutamyl transferase and bilirubin in athletes after a completing a marathon (n=17), 100 km ultra-marathon (n=17) and 308 km ultra-marathon (n=16). Alkaline phosphatase was significantly elevated following the marathon, with bilirubin only significantly elevated after 100 km, while gamma-glutamyl transferase increased at marathon length and decreased thereafter. This profile may be associated with mild or transient hepatic damage that may correlate with distance. Although the notion of mild or transient hepatic damage is further supported by numerous studies (Table 1), this topic seems quite controversial as a select few contrarily reported pronounced and/or prolonged damage (Carvalho *et al.*, 2016; Khan *et al.*, 2018). Nevertheless, these changes unanimously correlate to distance/rate of distance covered, intensity and possibly age.

(4) Immune response and inflammation

The human immune system comprises of two major defence mechanisms namely the; (a) innate and (b) adaptive immune response. The innate immune response focuses on generic protection mechanisms which include localised inflammation, phagocytosis and major histone complex stimulation. The adaptive immune response is mainly enforced by lymphocytes (T-cells, B-cells and immunoglobulins

[Ig]) and is more antigen-specific, which is usually activated when the threat/cellular insult has not been neutralised by the innate system (Goh, Lim & Suzuki, 2019). Physical activity has a profound effect on the immune system (Nieman & Pence, 2019), however, the magnitude of this effect is generally thought to be modulated by the intensity and duration of exercise (Sellami *et al.*, 2018; Liu *et al.*, 2017a). Although moderate-intensity physical activity has been shown to improve the systemic immunity (Nieman, 2011), the intense physical stress of ER is associated with cellular damage, which leads to a cascade of inflammatory responses (Davison, Kehaya & Wyn Jones, 2016; Scheffer *et al.*, 2019). Inflammation is a key defence mechanism regulated by a complex network of pro- and anti-inflammatory cytokines/chemokines to prevent the prevalence of pathogenic diseases and/or internal damage induced by other harmful stimuli such as ER. These cytokines and chemokines may be naturally produced or ingested in the form of non-steroidal anti-inflammatory drugs, which prevents excessive tissue destruction and/or injury (Opal & DePalo, 2000). Excessive inflammation provokes the onset of numerous chronic diseases, including insulin resistance, cardiovascular disease and Alzheimer's disease (Liu, Wang & Jiang, 2017b; Opal & DePalo, 2000), only to name a few. Hence, a symbiotic relationship between antagonistic mechanisms (pro- and anti-inflammatory molecules) is required in order to maintain overall health (Barros *et al.*, 2017). In the event of intense endurance activity, inflammation is mainly ascribed to mechanical shearing, reactive oxygen species (ROS)-related damage and various metabolic adaptations. Various biochemical methods have been used to characterise the immunological adaptations induced by prolonged physical activity (as summarised in Table 1). Some of the most popular markers associated with inflammation include elevations of C-reactive protein (CRP) and innate immune response white blood cells (WBC; also known as leukocytes) including monocytes, eosinophils, neutrophils, basophils and macrophages (Table 1). CRP is produced and stored by hepatocytes and secreted in response to the elevated levels of pro-inflammatory cytokines. Despite structural dissimilarities, this marker possess similar functions to Ig, particular pertaining to phagocytosis, agglutination and component pathway stimulation (Shrivastava *et al.*, 2015) and is therefore used as an indication of the immune response against a plethora of diseases, such as atherosclerosis and recurrent ischemia (Shrivastava *et al.*, 2015). To this end, Zakovska *et al.* (2017) observed non-significantly (IgA and IgM) and significantly (IgG) elevated Ig in 15 athletes after

290 completing a 100 km ultra-marathon under freezing conditions. On the contrary, numerous studies
291 (Table 1) indicated a reduction or suppression of these immune cells and CRP production after ER or
292 exercise. Immunosuppression trends have been associated with a phenomenon known as the ‘open
293 window effect’ (OWE) (Bekos *et al.*, 2016). The OWE is based on the perception that immune function
294 may be impaired for an extended period after intensive exercise bouts (Goh *et al.*, 2019), increasing the
295 athlete’s susceptibility to diseases, particularly to upper respiratory tract infections (illustrated by the J-
296 curve). As such, the reduction in circulatory immune cells can be ascribed to the relocation of these
297 cells to peripheral tissue (Goh *et al.*, 2019; Nieman, 2000). However, hypotheses such as the J-curve
298 and the OWE have been challenged by various studies (Robson-Ansley *et al.*, 2012; Mach & Fuster-
299 Botella, 2017), possibly debunking the relationship of acute bouts of exercise and immunosuppression
300 (Campbell & Turner, 2018), and giving rise to an alternative model known as the ‘tissue damage or
301 injury model’ (TIM). The TIM proposes that immunosuppression and the subsequent susceptibility to
302 illnesses arise from imbalanced pro- and anti-inflammatory regulation due to an increased training load
303 and frequency, accompanied by insufficient system recovery (Goh *et al.*, 2019). This model also places
304 emphasis on the production of alarmins (damage-associated molecular patterns) in response to exercise-
305 induced tissue or cellular insults, such as apoptosis and necrosis, subsequently stimulating cytokine and
306 chemokine distribution (Goh *et al.*, 2019; Yang, Han & Oppenheim, 2017). This was supported by
307 Bekos *et al.* (2016) who investigated the biochemical adaptations of the receptor of advanced glycation
308 end products (RAGE) and suppression of tumorigenicity factor 2, in athletes following a half-marathon
309 (n=36) or marathon (n=34), in comparison to sedentary volunteers (n=30). Serum and urine
310 measurements were collected at baseline, the finish line as well as during recovery, and indicated
311 elevated concentrations of high mobility group box 1 alarmins, advanced glycated end-products (AGE)
312 carboxymethyl-lysine, and soluble receptor of advanced glycation end-products (RAGE; ligands of
313 AGE), accompanied by altered cytokeratin 18 (CK18) concentrations. Although serum CK18 was
314 elevated in both marathon and half-marathon runners, urinary CK18 was exclusively elevated in the
315 half-marathon participants, whereas caspase-cleaved cytokeratin (ccCK18) remained unchanged in all
316 three cohorts and sample media. Since ccCK18 is only produced as a result of apoptosis of hepatic and
317 renal cells (Li *et al.*, 2017), the isolated elevation in CK18 (parent molecule), may indicate the

318 predominant prevalence of cellular necrosis induced by inflammation rather than apoptosis (Bekos *et*
 319 *al.*, 2016). In addition to the leucocyte and lymphocyte production and stimulation, cellular necrosis
 320 releases various molecules (such as the aforementioned alarmins) in to circulation, which is crucial for
 321 cytokine and chemokine stimulation (Goh *et al.*, 2019), creating a positive feedback regulation loop
 322 and the subsequent increase in cellular destruction.

323 Nielsen *et al.* (2016) investigated cytokine profiles of 40 individuals participating in endurance
 324 activities (marathon, half-marathon and a ranger-training course) and observed a substantial increase in
 325 interleukin (IL)-6, -8 and -10, as a result of increased WBC counts following muscle shearing, and
 326 possibly also the activation of catecholamines (dopamine, epinephrine and norepinephrine), as
 327 described by Arakawa *et al.* (2016). However, numerous cytokines, such as tumour necrosis factor- α
 328 (TNF- α), interferon-gamma (IFN- γ), vascular endothelial growth factor, IL-2, -4, -5, -12, and -1 β ,
 329 remained almost unchanged in both ER cohorts, which is ascribed to the complex regulation of pro-
 330 and anti-inflammatory cytokines (Nielsen *et al.*, 2016). It was further suggested, that the significant
 331 accumulation of the multi-functional IL-6 (pro-inflammatory cytokine and anti-inflammatory myokine)
 332 and anti-inflammatory IL-10, might have intercepted the production of TNF- α and IL-1 β (Nielsen *et*
 333 *al.*, 2016), leading to is reduction. On the contrary, however, numerous studies (Table 1) have reported
 334 significant alterations in the aforementioned cytokines. Sierra *et al.* (2019) investigated the impairment
 335 of cardiopulmonary functionality in 31 male athletes after completing a marathon and observed a
 336 negative correlation between cardiopulmonary function and acute inflammatory markers. Significant
 337 reductions in the sputum (which was induced) IL-12p40, IL-23, IL-33 and thymic stromal
 338 lymphopoietin, accompanied by elevated neutrophils, macrophages, epithelial cells and nitric oxide
 339 exhalation was observed immediately after the marathon. Although these results indicate increased
 340 airway inflammation, the reduction of the aforementioned cytokines diminish the differentiation of T-
 341 helper cells (Th) 1, Th2, and Th17, suggesting an imbalanced airway immune response, as well as the
 342 possible impediment of cardiopulmonary abilities (Sierra *et al.*, 2019). In light of pulmonary function
 343 and inflammation induced by ER, Bekos *et al.* (2019) investigated the prevalence of exercise-induced
 344 bronchoconstriction and the role of heat shock proteins (HSPs) in non-asthmatic half-marathon and

marathon recreational athletes. HSPs are multi-functional proteins that assist with protein folding, translocation and synthesis, and also act as key regulatory factors secreted in response to stressors such as temperature fluctuations, increased ROS production, inflammation, etc. (Santoro, 2000; Garrett & Grisham, 2013). Significantly elevated concentrations of HSP70 and HSP27 were observed immediately after the ER, however, cohort comparisons indicated larger accumulations of these HSPs in marathon athletes. As such, HSPs, in particular HSP70, is possibly upregulated due to increased ROS, inflammation and environmental stress induced by ER. However, since these correlate with the fluctuation of WBC concentrations in the athletes, it may also be indicative of a predisposition to exercise-induced bronchoconstriction.

Considering the above, it is clear that inflammation and the overall immune response to ER are not only dependent on, or influenced by, the complex nature of immune regulation, but also the duration and nature of exercise (Elkington *et al.*, 2015; Nieman, Lila & Gillitt, 2019). When considering the literature (Table 1), one could also speculate that cohort fitness, age and sex influence inflammatory marker fluctuations.

(5) Structural damage and associated hormones

Kim *et al.* (2007) investigated the mechanical implications of ER by measuring the alterations in cartilage and muscle damage-associated markers (amongst others) in 54 trained athletes, after a 200 km run. A three-fold increase in cartilage oligomeric matrix protein (COMP) indicated notable cartilage damage, especially during the second half (>100 km) of the race. This was accompanied by elevated creatine kinase (19-fold at 100 km and 90-fold at 200 km) and numerous cytokine markers, which were evident of substantial microfibrillar (a muscle constituent) damage, substantiating the findings discussed in Section II.3. Cartilage damage associated with ER was confirmed by Mündermann *et al.* (2017), who observed elevated COMP levels, even at shorter distances (i.e. marathon length and training distances), in conjunction with elevated heat sensitive-CRP concentrations post-race, both of which correlated with athlete finishing times. Contradictory, Vuolteenaho *et al.* (2014) observed unchanged COMP concentrations in 46 male athletes after completing a marathon, suggesting its dependence on factors other than distance and speed, such as athlete sex, training or fitness status, and

the rational of athlete participation (competitive or non-competitive). Furthermore, Vuolteenaho *et al.* (2014) also investigated ER-induced fluctuations in other cartilage damage-associated markers, such as metalloproteinase-3 (MMP-3), chitinase 3-like protein (YKL-40), and inflammatory-associated adipokines (specifically adiponectin, leptin and resistin). Elevations in MMP-3 and YKL-40 depicted the severe effects of ER on articular cartilage since these markers are regularly observed in patients diagnosed with osteoarthritis (Sharif, Malaise & De Seny, 2014). The fluctuations in MMP-3 directly correlated with the finishing times of athletes (where faster athletes had higher MMP-3 levels), however, no such correlation could be made with COMP and YKL-40. Significantly elevated adiponectin and resistin, accompanied by the non-significant reduction in leptin, were mainly ascribed to increased inflammatory activity in response to cartilage damage. However, only resistin could be positively correlated with cartilage damage-associated markers, suggesting that resistin may actually be a part of cartilage catabolism during ER. Additionally, considering that resistin is produced by numerous cell types, including osteoblasts (bone formation) and osteoclasts (bone resorption), elevations thereof may also be indicative of adaptations in athlete bone metabolism (Thommesen *et al.*, 2006). Extensive mechanical loading consisting of repeated structure straining and temporary relief thereof (as in the case of ER) has been reported to inflict micro-fatigue damages to bone structures (Kersch-Schindl *et al.*, 2009), collagen type 1 destruction (Sansoni *et al.*, 2017), and adaptations in bone metabolism (Mouzopoulos *et al.*, 2007). This is supported by Kersch-Schindl *et al.* (2015) in a study aimed at determining the effects of a 246 km ultra-marathon on numerous musculoskeletal markers including dickkopf-1, sclerostin, cathepsin, pro-collagen type I N-terminal pro-peptide (P1NP), cross-linked-C-telopeptide of type I collagen (CTX), myostatin and follistatin. The elevated concentrations of both myostatin (inhibition of muscle growth) and its antagonist, follistatin (activation of muscle growth), immediately after the race may be accredited to the dysregulation of muscle metabolism associated extreme overreaching (as in the case of ER). However, the authors also annotated that these elevations could be indicative of rhabdomyolysis (Section II.3) and the fluctuation in haem concentrations associated with ER (Section II.1). Additionally, a significant reduction in P1NP (bone formation marker), accompanied by elevated CTX (bone resorption marker), were observed immediately post-race and ascribed to temporary uncoupled bone metabolism (Kersch-Schindl *et al.*,

2015). Although not observed in this study, it is worth noting, that these bone turnover markers seem to be affected by or dependent on, factors such as age, pregnancy, sex, medication and previous skeletal fractures (Szulc *et al.*, 2017). In addition, reductions in sclerostin (non-significant) and dickkopf-1 could signify an initial activation of osteoblast anabolism since both markers are inhibitors of the Wnt-pathway, responsible for osteoblast commitment/differentiation. However, this profile has also been attributed to possible targeted remodelling and repair of micro-damage (Kersch-Schindl *et al.*, 2015). Furthermore, Kersch-Schindl *et al.* (2009) also observed elevated concentrations of receptor activator of nuclear factor κ B ligand (RANKL) and osteoprotegerin, accompanied by reductions in osteocalcin, within 15 min after completing a 246 km ultra-marathon. Osteoprotegerin and RANKL are natural opponents, considering that RANKL is an activator of osteoclast cells, while osteoprotegerin acts as a decoy receptor for RANKL, thus inhibiting osteoclast production, and in effect bone resorption (Tobeiha *et al.*, 2020). Moreover, a reduction in the hormone osteocalcin indicated an overall reduction in bone turnover (Shin *et al.*, 2012; Mouzopoulos *et al.*, 2007; Sansoni *et al.*, 2017). Although ER acutely reduces bone formation/turnover — classifying ER as a negative influence on bone metabolism — this topic remains controversial amongst scientists as some studies have suggested that ER is beneficial in terms of bone turnover and cartilage health in the long-run, as in the case of Lee (2019). In this study, the effect of continuous ER on bone strength and biochemistry was investigated by comparing 68 athletes (recently enrolled in a marathon club) to 40 sedentary individuals. Although no significant difference was observed in deoxypyridinoline (pyridium-links found in collagen type I), a substantial increase in osteocalcin suggests a positive association between endurance activity and bone formation. These findings were intensity and duration dependent, however, athlete sex could also be a contributing factor since female athletes have a higher susceptibility to low mineral bone densities as a part of triad syndrome developed from with chronic ER (Daily & Stumbo, 2018).

Nevertheless, the acute effects of ER on bone metabolism, have also been linked to the perturbed homeostasis of other hormones besides osteocalcin. Mouzopoulos *et al.* (2007) evaluated bone turnover markers, cortisol and parathyroid hormone in 16 athletes after the completion of a 245 km ultra-marathon. Elevated cortisol and parathyroid hormone correlated with reduced osteocalcin

concentrations, immediately after the race. Since cortisol and parathyroid hormone reportedly reduce or suppress osteoblast proliferation and function, reduced osteocalcin may result from the elevation of these hormones. Additionally, Sansoni *et al.* (2017) observed fluctuations in adipokines (visfatin, resistin and leptin) and various other hormones (insulin, glucagon, ghrelin, C-peptide, glucagon-like peptide 1 [GLP-1] and gastric inhibitory polypeptide [GIP]) in 17 male athletes, after a mountain ultra-marathon (65 km). Although adipokines are well-known signalling molecules, secreted as a result of various immune responses, some possess the ability to function as metabolic hormones associated with the regulation of fuel substrate catabolism, appetite stimulation, apoptosis, emotional well-being, as well as cellular proliferation and growth (Kurotani *et al.*, 2017). Considering that many of these hormones are related to immune response activation, fluctuations may arise as a result of bone or collagen destruction. However, besides the fact that running extensive distances induces various fluctuations in fuel substrate metabolisms (Section III), bone remodelling is considered to be an energy taxing process which may also contribute to aforementioned hormone changes observed. Evidence of the intertwined nature of this is portrayed by the association between resistin and fat oxidation, visfatin and the regulation of glucose metabolism, nicotinamide adenine dinucleotide (NAD⁺) production and odd-chain fatty acids (OCFA) (Kurotani *et al.*, 2017), as well as leptin and energy homeostasis (Hackney, 2019; Garrett & Grisham, 2013). As such, the aforementioned fluctuations in adipokines and metabolic hormones were associated with increased glucose utilisation (discussed in Section III.1), deregulated insulin mechanisms, and inflammatory response (Sansoni *et al.*, 2017).

III. Metabolomic biomarkers

The human metabolome collectively refers to all small intermediates (>1.5 kDa; metabolites) that participate in the metabolic reactions needed for cell function, growth and maintenance (Rieckeberg & Powers, 2017; Nieman *et al.*, 2019). Since the metabolome is a reflection of the genome, transcriptome and proteome, intervention of a stimulus at any of these levels will result in characteristic metabolic changes (Stipetic *et al.*, 2016), subsequently providing a representation of the organism's overall physiological status (Turkoglu *et al.*, 2016). The science of identification and quantification of these metabolites, using highly selective and sensitive analytical instruments, in conjunction with

biostatistical analyses, is commonly referred to as metabolomics (De Villiers & Loots, 2013). Considering the extensive energy-requirements necessary for completing ER events, as well as the physiological and pathological impact that these races have on the human body, metabolomics can be considered a formidable tool when attempting to better characterise the effects of ER.

Energy (in the form of adenosine triphosphate [ATP]) expenditure during physical activity can be described in a hierarchical manner, consisting of three main phases that are mainly dependent on the duration and intensity of the physical activity (MacLaren & Morton, 2012). The first/primary phase consists of phosphocreatine catabolism (intramuscular ATP stores) and provides sufficient energy for approximately 1–10 s at high intensities (MacLaren & Morton, 2012). During the second phase, anaerobic glycolysis is activated to produce lactate and subsequently ATP (Salway, 2012), for approximately 10–60 s of the high-intensity exercise (MacLaren & Morton, 2012). The first two phases provide limited amounts of ATP, while phase three provides the energy required for high-intensity exercise exceeding 60 s via aerobic fuel substrate (carbohydrate, lipid and amino acid [AA]) catabolism (MacLaren & Morton, 2012). As such, most of the studies investigating the effects of endurance exercise on the metabolome of athletes would indicate alterations within the aforementioned fuel substrate catabolic pathways. However, a large portion of the available literature to date is focused on the metabolic adaptations induced by various endurance activities, including rowing (Yan *et al.*, 2009), weightlifting (Sheedy *et al.*, 2014), cycling and ergonomic cycling (Peake *et al.*, 2014; Nieman *et al.*, 2014; Chorell *et al.*, 2012), repeated sprint bouts (Pechlivanis *et al.*, 2010; Stathis *et al.*, 2006; Pechlivanis *et al.*, 2013), voluntary leg extension exercises (Costa *et al.*, 2015), as well as team sports, including rugby (Dubois *et al.*, 2017), leaving a certain void for such studies focused on ER specifically. To this end, Table 2 provides a summary of the studies (published 2000–2020) focused on the altered human metabolism induced by ER, with the exclusion of those that are based on animal models and/or the effects of supplementation, since these fall beyond the scope of this review. In the remainder of this review, the metabolic changes induced by ER will be discussed in context of the major metabolic fuels and associated pathways affected during ER and the factor that might influence it.

480 **Table 2:** Endurance running-induced metabolic biomarker adaptations reported in recent (2000–2020) literature, arranged according to the publication year.

Reference	Exercise type	Cohort	Sampling media	Metabolic adaptations	
Mastaloudis, Leonard & Traber (2001)	Ultra-marathon (50 km)	11 participants (8 male; 3 female)	Blood; fasting state, before, at 27 km, immediately after, 1 h ⁺ and 24 h ⁺ post-race	After 27 km of running: Lipid, ketones and cholesterol: Chol (–); TAG (↑)* ROS and vitamins: Urate (↑) ^a ; α-Tocopherol (↑) ^a ; Ascorbate (↑) ^a	Ultra-marathon length Lipid, ketones and cholesterol: Chol (–); TAG (↑)*,b; ROS and vitamins: Urate (↑) ^a ; α-Tocopherol (↑) ^a ; Ascorbate (↑) ^{a,b}
Kratz <i>et al.</i> (2002)	Marathon	37 participants (32 male; 5 female)	Blood; before, within 4 h after and 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Lipid, ketones and cholesterol: TAG (↓)*; Chol (↑)* ROS and vitamins: Urate (↑) ^a ; Cr (↑)	
Petibois <i>et al.</i> (2002)	10 km run	14 participants (<i>sex not provided</i>)	Blood; before and immediately post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a ; Lactate (↑) ^a Lipid, ketones and cholesterol: TAG (↓) ^a ; Glycerol (↑) ^a ; Urea (↑) ^a	
Uchakin <i>et al.</i> (2003)	Marathon (White Rock and Cowton marathons)	15 male participants	Blood; before, 20 min and 60 min after the race as well as 2 ⁺ , 5 ⁺ and 8-days ⁺ post-race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	
Niessner <i>et al.</i> (2003)	Marathon	19 participants (17 male; 2 female)	Blood; before and immediately after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	
Real <i>et al.</i> (2005)	Marathon	22 male participants	Blood; before and after the marathon	Post-race: Carbohydrates and TCA cycle: Glucose (–) Lipid, ketones and cholesterol: FA (↑) ^a ; TAG (↓) ^a Amino acids and urea cycle: Homocysteine (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ROS and vitamins: Folate (↑)*; Vit B12 (↓)*	
Leers <i>et al.</i> (2006)	Marathon	27 participants (25 male; 2 female)	Blood; before, immediately after and 24 h ⁺ post-race	Post-race: Amino acids and urea cycle: Urea (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a	
Kim <i>et al.</i> (2007)	Ultra-marathon (200 km)	54 male participants	Blood; before, after 100 km and immediately post-race	Post-race: Carbohydrates and TCA cycle: Lactate (↑)*; Glucose (↓)* Purine, pyrimidine and ATP: Cr (↑) ^a	

Mouzopoulos <i>et al.</i> (2007)	Ultra-marathon (245 km)	16 male participants	Blood; 5-days before, immediately after as well as 1 ⁺ , 3 ⁺ and 5-days ⁺ post-race	Post-race: Amino acids and urea cycle: OH-proline (↓) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a
Siegel <i>et al.</i> (2007)	Marathon	33 participants (<i>sex not provided</i>)	Blood; before and after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a
Weibel & Glonek (2007)	Ultra-marathon (>65 km per day for 6 days)	31 participants	Urine; before and 4-20 times during the race	Post-race (average): Lipid, ketones and cholesterol: Ketones (↑) ^a (n=22); Ketones (↓) ^a (n=9)
Lehmann <i>et al.</i> (2010)	Treadmill exercises (±14 km -16 km)	21 participants	Blood; before and immediately after exercise	Post-race: Carbohydrates and TCA cycle: Lactate (↑) ^a Lipid, ketones and cholesterol: Hippurate (↑) ^a ; C6:0 car. (↑) ^a ; C8:0 car. (↑) ^a ; C10:1 car. (↑) ^a ; C10:0 car. (↑) ^a ; C14:2 car. (↑) ^a ; C12:0 car. (↑) ^a
Lewis <i>et al.</i> (2010)	Marathon and 60 min treadmill running exercises	25 marathon participants and 45 individuals (treadmill exercise)	Blood; before and after exercise	Treadmill exercise: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Malate (↑) ^a ; Succinate (↑) ^a ; Fumarate (↑) ^a ; Pyruvate (↑) ^a ; Glucose-6-phosphate (↑) ^a ; 3-Phosphoglycerate (↑) ^a Lipid, ketones and cholesterol: Glycerol (↑) ^a Amino acids and urea cycle: Alanine(↑) ^a ; Leucine (↑) ^a ; Serine (↓)*; Glutamate (↓) ^a ; Cysteine (↓) ^a ; Homocysteine (↓) ^a ; Glutamine (↓) ^a ; Citrulline (↓) ^a Purine, pyrimidine and ATP: Inosine (↑) ^a ; Hypoxanthine (↑) ^a ; AMP (↑) ^a ; Xanthine (↑) ^a ; Allantoin (↓) ^a ; Niacin (↑) ^a ; Pantothenate (↑) ^a ; Cr (↑)* Marathon distance: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Malate (↑)*; Aconitate (↑) ^a ; α-ketoglutarate (↑) ^a ; Citrate (↑) ^a ; Succinate (↑) ^a ; Fumarate (↑) ^a ; Pyruvate (↑) ^a ; Glucose-6-phosphate (↑) ^a Lipid, ketones and cholesterol: Glycerol-3-phosphate (↑) ^a ; Glycerol (↑) ^a ; β-OH-butyrate (↑) ^a Amino acids and urea cycle: Leucine (↓) ^a ; Serine (↓) ^a ; Glutamine (↓) ^a ; Proline (↓) ^a ; Lysine (↓) ^a ; Threonine (↓) ^a ; Betaine (↓) ^a ; Asparagine (↓) ^a ; Dimethylglycine (↓) ^a ; Histidine (↓) ^a ; Kynurenate (↑) ^a ; Valine (↓) ^a ; Homovanillate (↑) ^a ; 4-OH-phenylpyruvate (↑) ^a ; Arginine (↓) ^a ; Quinolate (↑) ^a ; Tryptophan (↓) ^a ; Citrulline (↓) ^a ; Ornithine (↓) ^a ; Argininosuccinate (↑) ^a Purine, pyrimidine and ATP: Hypoxanthine (↑) ^a ; AMP (↑) ^a ; IMP (↑) ^a ; Xanthine (↑) ^a ; Xanosine (↑) ^a ; Niacin (↑) ^a ; Pantothenate (↑) ^a ; Cr (↑)*
McCullough <i>et al.</i> (2011)	Marathon	25 participants (12 male; 13 female)	Blood: before, after and 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a

Turner <i>et al.</i> (2011)	Ultra-marathon (233 km)	9 male participants	Blood; before, immediately after as well as 24 h ⁺ , 7 ⁺ and 28-days ⁺ post-race	Post-race: ROS and vitamins: Glutathione (↑) ^a ; Lipid peroxides (↑)		
Waśkiewicz <i>et al.</i> (2012)	Ultra-marathon (168.5 km)	19 male participants	Blood; before, at marathon distance, within 12 h and immediately after the ultra-marathon	Post-race: Lipid, ketones and cholesterol: Chol (–); TAG (–); FA (↑) ^a ; Glycerol (↑) ^a ; β-OH-butyrate (↑) [*]	12 h of running: Lipid, ketones and cholesterol: Chol (–); TAG (–); FA (↑) ^{a,b} ; Glycerol (↑) ^{a,c} ; β-OH-butyrate (↑) ^{a,c}	Post-race: Lipid, ketones and cholesterol: Chol (↓) ^a ; TAG (↓) ^a ; FA (↑) ^{a,c} ; Glycerol (↑) ^{a,c} ; β-OH-butyrate (↑) ^{a,b}
Klapcinska <i>et al.</i> (2013)	Ultra-marathon (48 h; 183–320 km)	7 male participants	Blood; before, after running 12 h, 24 h and within 10 min after the race as well as 24 h ⁺ and 48 h ⁺ post-race	12 h of running: Carbohydrates and TCA cycle: Lactate (↓) [*] ; Glucose (↓) [*]	24 h of running: Carbohydrates and TCA cycle: Lactate (↓) ^{*,c} ; Glucose (↑) [*]	Post-race: Carbohydrates and TCA cycle: Lactate (↓) ^{*,c} ; Glucose (↑) ^{*,b}
Nieman <i>et al.</i> (2013)	Repeated treadmill exercises (±25 km per day for 3 days)	15 participants (7 male; 8 female)	Blood; before and directly after the 3-day intensified training exercise	Post-exercise: Carbohydrates and TCA cycle: Succinate (↑) ^a ; Fumarate (↑) ^a ; Malate (↑) ^a Lipid, ketones and cholesterol: Palmitoleate (↑) ^a ; Myristoleate (↑) ^a ; 10-heptadecenoate (↑) ^a ; 10-nonadecenoate (↑) ^a ; Dihomolinoleate (↑) ^a ; Stearidonate (↑) ^a ; Myristate (↑) ^a ; Eicosenoate (↑) ^a ; Oleate (↑) ^a ; Margarate (↑) ^a ; Laurylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; 4-decenoylcar. (↑) ^a ; Dodecanedioate (↑) ^a ; Hexadecanedioate (↑) ^a ; Tetradecanedioate (↑) ^a ; Octadecanedioate (↑) ^a ; Linolenate (↑) ^a ; Docosapentaenoate (↑) ^a ; Linoleate (↑) ^a ; Eicosapentaenoate (↑) ^a ; Dihomolinolenate (↑) ^a ; Docosahexaenoate (↑) ^a Lysolipid, bile salts and steroids/hormones: 1-arachidonoylGPEA (↓) ^a ; 1-palmitoylGPEA (↓) ^a ; 1-oleoylGPEA (↓) ^a ; 2-linoleoylGPEA (↓) ^a ; Taurodeoxycholate (↓) ^a ; Taurocholate (↓) ^a ; Glycochenodeoxycholate (↓) ^a ; Glycodeoxycholate (↓) ^a ; Cholate (↓) ^a Glycocholate (↓) ^a ; 2-aminoadipate (↑) ^a Amino acids and urea cycle: 4-OH-phenyl-pyruvate (↑) ^a ; N-acetylphenylalanine (↑) ^a ; Isovalerylcar. (↑) ^a ; OH-isobutyrate (↑) ^a		
Daskalaki <i>et al.</i> (2015)	1 h treadmill/bicycle ergometer exercise	3 male participants	Urine; before (first pass) ⁺ , 1 h before ⁺ , immediately after as well as numerous time s within the	Post-exercise: Carbohydrates and TCA cycle: Pyruvate (↑) ^a Purine, pyrimidine and ATP: N2-N2-Dimethylguanosine (↑) [*] ; Xanthosine (↑) ^a ; Inosine (↑) ^a ; Deoxyinosine (↑) ^a ; Guanine (↑) ^a ; Hypoxanthine (↑) ^a Amino acids and urea cycle: 3-OH-tryptophan (↑) [*] ; Xanthurenate isomer (↑) ^a ; Kynurenate (↑) [*] ; OH-tryptophol (↓) [*] ; N1-Methyl-2-pyridone-5-carboxamide (↑) [*]		

			preceding 24 h ⁺ post-exercise	Tryptophan (↓)*; O-Acetyl-L-homoserine (↑)^a; Histidine (↓)*; Methionine (↑)*; Proline (↑)*; Acetylvanilalanine (↑)^a; N-(Carboxyethyl) arginine (↑)^a; N-Acetyl-D-tryptophan (↑)^a; N-acetylmethionine (↑)*; Indole pyruvate (↑)*; Acetamido-oxohexanoate (↑)*; OH-phenylpyruvate (↑)*; Oxoarginine (↑)*; Guanidovaleramide (↑)^a; AcetoOH-butanoate (↑)^a; Acetamidobutanoate (↑)*; DiOH-methylbutanoate (↑)*; Methyl-oxopentanoate (↑)*; Dioxopentanoate (↑)*; OH-pentanoate (↑)*; Methyloxobutanoate (↑)*; OH-butanoate(↑)* ROS and vitamins: Riboflavin (↑)*; Pantothenate (↑)^a Lipid, ketones and cholesterol: Dodecenoylcar. (↑)^a; Undecanoylcar. (↑)*; Decanoylcar. (↑)*; L-Octanoylcar. (↑)*; Methylglutarylcar. (↑)*; Hexanoylcar. (↑)*; Valerylcar. (↑)*; DeOH-car. (↑)^a Lysolipid, bile salts and steroids/hormones: Urocortisol glc. (↑)*; Dihydrocortisone glc. (↑)*; Hydrocortisone sulphate (↑)*; OH-androsterone glc. (↑)*; Androstane diol glc. (↑)*; Androsterone glc. (↑)*; Oxoandrostane glc. (↑)*	
Khodaei <i>et al.</i> (2015)	Ultra-marathon (161 km)	20 participants pre-marathon (14 male; 6 female), 21 participants post-marathon (17 male; 4 female)	Blood; before and immediately after	Post-race: Carbohydrates and TCA cycle: Glucose (↑)^a	
Mrakic-Sposta <i>et al.</i> (2015)	Ultra-marathon (330 km)	25 male participants	Blood and urine; before, during (±165 km) and immediately post-race	After 165 km of running: Carbohydrates and TCA cycle: Lactate (↑)^a; Glucose (↑)* Lipid, ketones and cholesterol: Ketones (–) Purine, pyrimidine and ATP: Cr (–) ROS and vitamins: Neopterin (–); ROS production rate (↑)^b; 8-OH-2-deoxyguanosine (–); 8-isoprostane (–)	Post-race: Carbohydrates and TCA cycle: Lactate (↑)^a; Glucose (↑)* Lipid, ketones and cholesterol: Ketones (↑)^a Purine, pyrimidine and ATP: Cr (↑)^a ROS and vitamins: Neopterin (↑)^a; ROS production rate (↑)^a; 8-OH-2-deoxyguanosine (↑)^a; 8-isoprostane (↑)^a
Suzuki <i>et al.</i> (2015)	5 h running exercise (Participant A= 34.14 km; participant B=43.05 km)	2 female participants	Blood; before, within 60 min after as well as 1 ⁺ , 3 and 5 h post-race	Participant A post-race: Carbohydrates and TCA cycle: Glucose (↑) Lysolipid, bile salts and steroids/hormones: Cortisol (↓); Adrenalin (↑); Noradrenalin (↑); Dopamine (↑) Lipid, ketones and cholesterol: TAG (↓), Acetoacetate (↑), 3-OH-butyrate (↑) Amino acids and urea cycle: Aspartate (↓); Glutamate (↑); Asparagine (↑); Serine (↑); Glutamine (↑); Glycine (↑); Histidine (↑); Arginine (↑); Taurine (↓); Threonine (↓); Alanine (↑); Proline (↑); Tyrosine (↑); Valine (↑);	Participant B post-race: Carbohydrates and TCA cycle: Glucose (↑) Lysolipid, bile salts and steroids/hormones: Cortisol (↑); Adrenalin (↑); Noradrenalin (↑); Dopamine (↑) Lipid, ketones and cholesterol: TAG (↓), Acetoacetate (↑), 3-OH-butyrate (↑) Amino acids and urea cycle: Aspartate (↓); Glutamate (↓); Asparagine (↓); Serine (↓); Glutamine (↓); Glycine (↓); Histidine (↓); Arginine (↓); Taurine (↓); Threonine (↓); Alanine (↓); Proline (↓); Tyrosine (↑); Valine (↓);

				Methionine (↑); Isoleucine (↑); Leucine (↑); Phenylalanine (↑); Lysine (↑)	Methionine (↑); Isoleucine (↓); Leucine (↑); Phenylalanine (↓); Lysine (↓)
Arakawa <i>et al.</i> (2016)	Ultra-marathon (130 km)	18 middle-aged participants	Blood; before, after 42 km (marathon), immediately after as well as 3 ⁺ , 5 ⁺ and 7-days ⁺ post-race	Marathon length: Lipid, ketones and cholesterol: FA (↑); TAG (↓)*; Chol (↑)*; HDL-C (↑)* Lysolipid, bile salts and steroids/hormones: Cortisol (↑)*; Adrenalin (↑) ^a ; Noradrenalin (↑) ^a ; Dopamine (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)	Post-race: Lipid, ketones and cholesterol: FA (↑) ^a ; TAG (↓) ^a ; Chol (↓); HDL-C (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a ; Adrenalin (↑) ^{a,b} ; Noradrenalin (↑) ^{a,c} ; Dopamine (↑) ^c Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*
Bekos <i>et al.</i> (2016)	Marathon and half-marathon	34 marathon participants, 36 half-marathon participants and 30 sedentary controls	Blood and urine; before and immediately after as well as 2–7 days ⁺ post-race	Half-marathon: Carbohydrates and TCA cycle: Lactate (↑) ^a Purine, pyrimidine and ATP: Urine Cr (–); Serum Cr (↑) ^a	Marathon: Carbohydrates and TCA cycle: Lactate (↑) ^a Purine, pyrimidine and ATP: Urine Cr (–); Serum Cr (↑) ^a
Shin <i>et al.</i> (2016)	Ultra-marathon (308 km)	15 participants (<i>sex not provided</i>)	Blood; before, at marathon distance, after 100 km and immediately post-race	Marathon length: Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) ^a	100 km of running: Purine, pyrimidine and ATP: Cr (↑) ^{a,d} ; Urate (↑) ^{a,d} Post-race: Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*
Traiperm <i>et al.</i> (2016)	Marathon	50 adolescent participants (30 male; 20 female)	Blood; before, immediately after as well as 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Lipid, ketones and cholesterol: TAG (↓) ^a ; HDL (↓) ^a ; LDL (↓) ^a ; Chol (↓) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*	
Vezzoli <i>et al.</i> (2016)	Ultra-marathons (50 km and 100 km)	24 participants (50 km n=6 [3 male; 3 female] and 100 km n=18 [7 male; 11 female])	Blood; before and immediately after the race	After 50 km: ROS and vitamins: Plasma: Cysteine (↑)*; Homocysteine (↑)*; Cysteinylglycine (↑)*; Glutathione (↑)*; TBARS (↑) ^a ; ROS (↑) ^a ; Erythrocyte: Cysteine (↑)*; Homocysteine (↑)*; Cysteinylglycine (↑)*; Glutathione (↑)*; 8-OH-2-deoxyguanosine (↑) ^a ; 8-isoprostane (↑) ^a	After 100 km: ROS and vitamins: Plasma: Cysteine (↑) ^a ; Homocysteine (↑) ^a ; Cysteinylglycine (↑) ^a ; Glutathione (↑) ^a ; TBARS (↑) ^a ; ROS (↑) ^a ; Erythrocyte: Cysteine (↑)*; Homocysteine (↑)*; Cysteinylglycine (↓)*; Glutathione (↑)*; 8-OH-2-deoxyguanosine (↑) ^a ; 8-isoprostane (↑) ^a
Yamada <i>et al.</i> (2016)	Ultra-marathon (135 km)	35 male participants	Blood; before, after 45 km and immediately post-race	45 km of running: Lipid, ketones and cholesterol: FA (↑) ^a Amino acids and urea cycle: Tryptophan (↓)*; Serotonin (↑) ^a ; Kynurenine (↓)*	Post-race: Lipid, ketones and cholesterol: FA (↑) ^{a,d} Amino acids and urea cycle: Tryptophan (↓) ^a ; Serotonin (↓)* ^c ; Kynurenine (↑)* ^d
Nieman, Sha & Pappan (2017)	Treadmill exercise (±24.9 km)	24 male participants	Muscle biopsy and blood; before and after exercise	Post-exercise: Carbohydrates and TCA cycle: Glycogen (↓) ^a ; Succinate (↑) ^a ; Malate (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a ; Adrenalin (↑) ^a	

				Lipid, ketones and cholesterol: Glycerol (↑) ^a ; Myristate (↑) ^a ; Oleate (↑) ^a ; Margarate (↑) ^a ; 16-OH-palmitate (↑) ^a ; 10-heptadecenoate (↑) ^a ; Myristoleate (↑) ^a ; Palmitoleate (↑) ^a ; Palmitate (↑) ^a ; Dihomolinoleate (↑) ^a ; Eicosenoate (↑) ^a ; Laurate (↑) ^a ; 10-nonadecenoate (↑) ^a ; Linoleate (↑) ^a ; Linolenate (↑) ^a ; Hexadecanedioate (↑) ^a ; Stearate (↑) ^a ; Docosadienoate (↑) ^a ; Nonadecanoate (↑) ^a ; 5-dodecenoate (↑) ^a ; Stearidonate (↑) ^a ; Arachidate (↑) ^a ; Pentadecanoate (↑) ^a ; 3-OH-butyrate (↑) ^a ; Caprate (↑) ^a ; Decanoylcar. (↑) ^a ; Methionylalanine (↑) ^a ; Tetradecanedioate (↑) ^a ; Octanoylcar. (↑) ^a ; Acetoacetate (↑) ^a ; 3-OH-decanoate (↑) ^a ; Docosapentaenoate (↑) ^a ; 13/9-HODE (↑) ^a ; 17-methylstearate (↑) ^a ; Laurylcar. (↑) ^a ; Decenoylcar. (↑) ^a ; Adrenate (↑) ^a ; Octadecanedioate (↑) ^a ; Erucate (↑) ^a ; 10-undecenoate (↑) ^a ; Methylpalmitate (↑) ^a ; 3-OH-octanoate (↑) ^a ; Dodecanedioate (↑) ^a
Da Ponte <i>et al.</i> (2018)	Marathon (up-hill only)	22 male participants	Blood; before and immediately post-race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a
Danese <i>et al.</i> (2017)	Half-marathon	30 participants (21 male; 9 female)	Blood; before and immediately after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cholate (↓) ^a ; Deoxycholate (↓) ^a ; Chenodeoxycholate (↓) ^a ; Ursodeoxycholate (↓) ^a ; Taurochenodeoxycholate (↑)*; Glycocholate (↓)*; Glycochenodeoxycholate (↓)*; Glycoursodeoxycholate (–); Glycodeoxycholate (–); Hyodeoxycholate (↓) ^a ; Lithocholate (–); Taurocholate (–)
Oliveira <i>et al.</i> (2017)	Marathon (hot and temperate temperatures)	26 male participants	Blood and urine, before, immediately after as well as 1 ⁺ and 3-days ⁺ after	Post-race (Heat): Amino acids and urea cycle: Urea (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) ^a ; TBARS (↑) ^a ; SOD3 (↑) ^a ; Catalase (↓)* Post-race (Temperate): Amino acids and urea cycle: Urea (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*; TBARS (↑) ^a ; SOD3 (↓)*; Catalase (↓)*
Davison <i>et al.</i> (2018)	1 h treadmill exercise (75% VO _{2max} ; Hypoxic and normoxic conditions)	24 male participants	Blood; before, immediately after as well as 3 h post-exercise ⁺	Post-exercise (Hypoxia): Lipid, ketones and cholesterol: Propionylcar. (↑) ^a ; Butyrylcar. (↑) ^a ; 2-Methylbutyrylcar./Pivaloylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Undecenoylcar. (↑) ^a ; 4,8 dimethylnonanoyl car. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Tridecenolcar. (↑) ^a ; Tetradecadienoylcar. (↑) ^a ; cis-5-Tetradecenoylcar. (↑) ^a ; Hexadecatetraenoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadec-2-enoyl car. (↑) ^a ; Palmitoylcar. (↑) ^a ; Octadecatrienoylcar. (↑) ^a ; Elaidic car./Vaccenyl car. (↑) ^a ; Hippurate(↑) ^a ; Myristate (↑) ^a ; Palmitoleate (↑) ^a ; α-Linolenate (↑) ^a ; Linoleate(↑) ^a ; Arachidonate (↑) ^a Post-exercise (Normoxia): Lipid, ketones and cholesterol: Propionylcar. (↑) ^a ; Butyrylcar. (↑) ^a ; 2-Methylbutyrylcar./Pivaloylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Undecenoylcar. (↑) ^a ; 4,8 dimethylnonanoyl car. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Tridecenolcar. (↑) ^a ; Tetradecadienoylcar. (↑) ^a ; cis-5-Tetradecenoylcar. (↑) ^a ; Hexadecatetraenoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadec-2-enoyl car. (↑) ^a ; Palmitoylcar. (↑) ^a ; Octadecatrienoylcar. (↑) ^a ; Elaidic car./Vaccenyl car. (↑) ^a ; Hippurate (↑) ^a ; Myristate (↑) ^a ; Palmitoleate (↑) ^a ; α-Linolenate (↑) ^a ; Linoleate (↑) ^a ; Arachidonate (↑) ^a

Howe <i>et al.</i> (2018)	80.5 km treadmill exercise (self-paced)	9 male participants	Blood; before and immediately post-exercise	Post-exercise: Carbohydrates and TCA cycle: Rhamnose (↓)^a; Galactosamine (↓)^a; Hexose (↓)^a Lipid, ketones and cholesterol: Acetylcar. (↑)^a; Propanoylcar. (↑)^a; Butanoylcar. (↑)^a; Hexenoylcar. (↑)^a; Hexanoylcar.isomer (↑)^a; Heptanoylcar. (↑)^a; Octenoylcar. (↑)^a; Octanoylcar. (↑)^a; Nonanoylcar. (↑)^a; Decadienoylcar. (↑)^a; Decenoylcar. (↑)^a; Decanoylcar. (↑)^a; Keto-decanoylcar. (↑)^a; Dimethylnonanoylcar. (↑)^a; Dodecenoylcar. (↑)^a; Hydroxydodecanoylcar. (↑)^a; Tetradecadienylcar.isomer (↑)^a; Tetradecanoylcar. (↑)^a; Hydroxytetradecadienylcar. (↑)^a; Hydroxytetradecenoylcar. (↑)^a; Hydroxymyristoylcar. (↑)^a; Hexadecadienoylcar. (↑)^a; Hexadecenoylcar. (↑)^a; Palmitoylcar. (↑)^a; Hydroxyhexadecadienoylcar. (↑)^a; Hydroxyhexadecenoylcar. (↑)^a; Octadecadienoylcar. (↑)^a; Hexadecanedioicacid monocar. ester (↑)^a; Decanoate (↑)^a; Dodecadienoate (↑)^a; Dodecanoate (↑)^a; Decanedioate (↑)^a; Hydroxydodecatrienolate; Tridecenoate (↑)^a; Tetradecadienoate (↑)^a; Tetradecenoate iso (↑)^a; Tetradecenoate iso (↑)^a; Dodecanedioate (↑)^a; Hydroxytetradecadienoate (↑)^a; Pentadecenoate (↑)^a; Hydroxytetradecadienoate (↑)^a; Hydroxytetradecanoate iso (↑)^a; Hydroxytetradecanoate iso (↑)^a; Hexadecadienoate iso (↑)^a; Hexadecadienoate iso (↑)^a; Palmitoleate (↑)^a; Tetradecanedioate (↑)^a; Hydroxyhexadecatrienoate (↑)^a; Hydroxyhexadecadienoate (↑)^a; Heptadecenoate (↑)^a; Hydroxyhexadecenoate iso (↑)^a; Hydroxyhexadecenoate (↑)^a; Hydroxyhexadecanoate (↑)^a; Octadecatetraenoate (↑)^a; Linolenate (↑)^a; Linoleate (↑)^a; Oleate (↑)^a; Dihydroxyhexadecadienoate (↑)^a; Dihydroxyhexdecenoate (↑)^a; Hydroxyoctadecadienoate (↑)^a; Hydroxyoctdecenoate (↑)^a; Dihydroxyoctadecanoate (↑)^a; Nitrooctadecenoate (↑)^a; Docosahexaenoate (↑)^a; Docosapentaenoate (↑)^a; Docosatetraenoate (↑)^a Amino acids and urea cycle: Glycine (↓)^a; Alanine (↓)^a; 3-Amino-isobutanoate (↓)^a; Serine (↓)^a; Pyrrole-2-carboxylate (↓)^a; Proline (↓)^a; Oxopentanoic acid (↓)^a; Guanidinoacetate (↓)^a; Valine (↓)^a; Betaine (↓)^a; Hydroxypentanoate (↑)^a; Threonine (↓)^a; Taurine (↓)^a; 5-Oxoproline (↓)^a; Hydroxyproline (↓)^a; Leucine (↓)^a; Isoleucine (↓)^a; Hydroxyhexanoic acid (↑)^a; Asparagine (↓)^a; Ornithine (↓)^a; Urocanate (↓)^a; Glutamine (↓)^a; Lysine (↓)^a; Glutamate (↓)^a; Methionine (↓)^a; Imidazol-5-yl-pyruvate (↓)^a; Indole-3-acetaldehyde (↓)^a; O-Acetylhomoserine (↓)^a; Arginine (↓)^a; Citrulline (↓)^a; Tyrosine (↓)^a; Hydroxyphenyllactate (↓)^a; Acetyl-L-lysine (↓)^a; Kynurenate (↑)^a; Tryptophan (↓)^a; Formylhydroxykynurenamine (↓)^a; Hydroxyindolepyruvate (↑)^a; 1-Methylnicotinamide (↓)^a Lysolipid, bile salts and steroids/hormones: Hydrocortisone (↑)^a; Urocortisone (↑)^a; Hydroxycholanate (↓)^a; Deoxycholanate (↓)^a; Chenodeoxyglycocholate (↓)^a; Glycocholate (↓)^a; Taurocholate (↓)^a; Chenodeoxycholate glc. (↓)^a; Cholestane—tetrol-glc. (↓)^a ROS and vitamins: γ-Tocopherol (↓)^a; α-Tocopherol (↓)^a Purine, pyrimidine and ATP: Hypoxanthine (↑)^a; Uridine (↓)^a; Pseudouridine (↓)^a
Li <i>et al.</i> (2018)	Ultra-marathon (400 km)	16 male participants	Blood; before and immediately after the race	Post-race: Amino acids and urea cycle: Alanine (↓)^a; Proline (↓)^a; Valine (↓)^a; Leucine (↓)^a; Isoleucine (↓)^a; Ornithine (↓)^a; Citrulline (↓)^a; Arginine (↑)[*]; Tryptophan (↓)[*]; Histidine (↑)[*]; Lysine (↓)^a; Methionine (↑)^a; Phenylalanine (↑)^a; Threonine (↓)^a; Glycine (↓)^a; Serine (↓)[*]; Taurine (↑)^a; Aspartate (↑)[*]; Glutamine (↑)[*]; Glutamate (↑)[*]; Cystathionine (↑)^a; Cystine (↑)^a; Homocysteine (—); 1-Methyl-L-

				histidine(↓)*; 3-Methyl-L-histidine (↑)*; α-Aminonbutyrate (↓)*; Argininosuccinate (↑) ^a ; β-Alanine (↑)*; Carnosine (↑) ^a ; Ethanolamine (↑)*; Homocitrulline (↓)*; OH-lysine (↓)*; Phosphorylethanolamine (↑) ^a ; Tyrosine (↑) ^a ; α-Aminoadipate (↑)*; Asparagine (↓)*; Phosphoserine (↓) ^a ; Sarcosin (↓) ^a Lysolipid, bile salts and steroids/hormones: Testosterone (↓) ^a ; Progesterone (↓) ^a ; 17-α-OH-progesterone (↓) ^a ; 11-Deoxycortisol (↓)*; 11-Deoxycorticosterone (↓)*; Cortisol (↑) ^a ; Aldosterone (↑) ^a ; Dehydroepiandrosterone (↓)*; Dehydroepiandrosterone sulphate (↑) ^a ; 4-Androstenedione (↓) ^a ; Dihydrotestosterone (↓)*; Corticosterone (↑)*; Cortisone (↓)*; Estrone (↑) ^a ; Oestradiol (↑)*
Stander <i>et al.</i> (2018)	Marathon	31 participants (19 male; 12 female)	Blood; before and immediately post-race	Post-race: Carbohydrates and TCA cycle: Arabitol (↑) ^a ; Citrate (↑) ^a ; Erythritol (↑) ^a ; Fumarate (↑) ^a ; Glucarate (↑) ^a ; Glucose (↑) ^a ; Malate (↑) ^a ; Mannitol (↑) ^a ; Mannose (↑) ^a ; Myo-inositol (↑) ^a ; Pyruvate (↑) ^a ; Sorbose (↑) ^a ; Succinate (↑) ^a ; Tagatose (↑) ^a ; Threonate (↑) ^a Lipid, ketones and cholesterol: α-OH-octanoate (↑) ^a ; α-Linolenate (↑) ^a ; β-OH-butyrate (↑) ^a ; OH-hexanoate (↑) ^a ; β-OH-α,β-didehydrosebacate (↑) ^a ; 5-Dodecenoate (↑) ^a ; 5-Pregnene-3β,20α-diol (↑) ^a ; 10-Heptadecenoate (↑) ^a ; 11-Eicosenoate (↑) ^a ; 11,14-Eicosadienoate (↑) ^a ; Acetoacetate (↑) ^a ; Glycerol (↑) ^a ; Heptadecanoate (↑) ^a ; Laurate (↑) ^a ; Linoleate (↑) ^a ; Malonate (↑) ^a ; Myristoleate (↑) ^a ; Oleate (↑) ^a ; Palmitate (↑) ^a ; Palmitoleate (↑) ^a ; Pentadecanoate (↑) ^a ; Monopalmitin (↑) ^a ; Squalene (↑) ^a ; Tridecanoate (↑) ^a Amino acids and urea cycle: α-Aminomalonate (↑) ^a ; α-Ethylhydracrylate (↑) ^a ; β-OH-isobutyrate (↑) ^a ; β-OH-isovalerate (↑) ^a ; β-OH-propionate (↑) ^a ; Alanine (↓) ^a ; Aspartate (↓) ^a ; Dimethylglycine (↓) ^a ; Glutarate (↓) ^a ; Glycerate (↑) ^a ; Glycine (↓) ^a ; Indole-3-acetate (↓) ^a ; Leucine (↓) ^a ; Methionine (↓) ^a ; Phenylalanine (↓) ^a ; p-OH-phenylacetate (↑) ^a ; p-OH-phenyllactate (↑) ^a ; Pyroglutamate (↓) ^a ; Serine (↓) ^a ; Threonine (↓) ^a ; Tyrosine (↓) ^a ; Valine (↓) ^a
Górecka <i>et al.</i> (2019)	Ultra-marathon	10 male participants	Blood; before, immediately after as well as 90 min post-race ⁺	Post-race: Lipid, ketones and cholesterol: Glycerol (↑) ^a ; TAG (↓) ^a ; Chol (↑)*; FA (↑) ^a
Malek <i>et al.</i> (2020)	Ultra-marathon (100 km)	18 participants (15 male; 3 female)	Blood; before and immediately post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑)*; Lactate (↑)*
Schader <i>et al.</i> (2020)	Marathon	76 male participants	Blood; before (one week), immediately after as well as 24 h ⁺ and 72 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Hexoses average (↓) ^a Lipid, ketones and cholesterol: C2:0–C18:0 car. (↑) ^a ; C2:1–C6:1 car. (↑) ^a ; C10:1–C18:1 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C14:2 car. (↑) ^a ; C14:2OH car. (↑) ^a ; C16:2 car. (↑) ^a ; C16OH car. (↑) ^a ; C18:2 car. (↑) ^a ; C18:1OH car. (↑) ^a ; C3OH car. (↑) ^a ; C5OH car. (↑) ^a ; C6 car. (↑) ^a ; LysoPC C14:0 (↓) ^a ; LysoPC C16:0 (↓) ^a ; LysoPC C16:1 (↓) ^a ; LysoPC C17:0 (↓) ^a ; LysoPC C18:0 (↓) ^a ; LysoPC C18:1 (↓) ^a ; LysoPC C18:2 (↓) ^a ; LysoPC C20:3 (↓) ^a ; LysoPC C20:4 (↓) ^a ; LysoPC C24:0 (↓) ^a ; LysoPC C28:0 (↓) ^a ; LysoPC C28:1 (↓) ^a ; PC C26–C44 (↓) ^a ; SMOH C14:1 (↓) ^a ; SMOH C16: (↓) ^a ; SMOH C22:1 (↓) ^a ; SMOH C22:2 (↓) ^a ; SMOH C24:1

				(↓) ^a ; SMOH C16:0 (↓) ^a ; SMOH C18:0 (↓) ^a ; SMOH C24:0 (↓) ^a ; SM C16:1 (↓) ^a SM C18:1 (↓) ^a SM C20:2 (↓) ^a SM C22:3 (↓) ^a SM C24:1 (↓) ^a SM C26:1 (↓) ^a Amino acids and urea cycle: Alanine (↓) ^a ; Arginine (↓) ^a ; Glutamine (↓) ^a ; Glutamate (↑) ^a ; Histidine (↓) ^a ; Leucine (↓) ^a ; Lysine(↓) ^a ; Methionine (↓) ^a ; Ornithine (↑) ^a ; Citrulline (↓) ^a ; Phenylalanine (↓) ^a ; Proline (↓) ^a ; Serine (↓) ^a ; Threonate (↓) ^a ; Tryptophan (↓) ^a ; Tyrosine (↓) ^a ; Valine (↓) ^a
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Footnote: Significance of adaptations are indicated as follows: significant (^a) and non-significant (*) compared to baseline; non-significantly/unreported elevations compared to preceding time point (^b); non-significantly/unreported reduction compared to preceding time point (^c); significantly elevated compared to preceding time point (^d), significantly reduced compared to preceding time point (^e), unchanged concentration (–), data not included in the table (⁺). **Abbreviations:** AMP: adenosine monophosphate; ATP: adenosine triphosphate; Car: carnitine; Chol: cholesterol; Cr: creatine; FA: fatty acids; Glc: glucuronide; GPEA: glycerophosphoethanolamine; HDL: high-density lipoprotein; Iso: isomer; LDL: low-density lipoprotein; OH: hydroxy; PC: Phosphatidylcholine; ROS: reactive oxygen species; SOD: superoxide dismutase; SM: Sphingomyelins; TAG: triacylglycerol; TBARS: thiobarbituric acid reactive substances; TCA: Tricarboxylic acid; Vit: vitamin.

(1) Carbohydrate metabolism and the tricarboxylic acid (TCA) cycle

Carbohydrates (muscle glycogen and glucose) are considered to be the primary fuel substrates utilised by the body during physical activity (Stellingwerff, 2012), making it a key component in the regulation of athletic performance. Glucose obtained or released from both intramuscular (muscle glycogen stores) and extra-muscular (liver glycogen and gastrointestinal absorption) sources are used as fuel substrates during physical activity, where the preference for usage depends on the intensity of exercise (MacLaren & Morton, 2012). As in the case of endurance exercise ($W_{max} \geq 75\%$), muscle glycogen accounts for most of the glucose catabolised. However, these stores are limited and may become ‘depleted’ (Hearris *et al.*, 2018; Nieman *et al.*, 2017) after approximately 90 min of exercise at $VO_{2max} \geq 75\%$ (Stellingwerff, 2012), affecting athletic performance. Lewis *et al.* (2010) employed a targeted (n=42 metabolites) liquid chromatography (LC)-mass spectrometry (MS) metabolomics approach and observed elevated concentrations of various glycolysis-associated intermediates (pyruvate, glucose-6-phosphate, lactate and niacinamide) in 25 athletes after a marathon. Elevated glycolysis intermediates detected immediately after ER indicates the upregulation of glucose catabolism, a finding which is supported by numerous other studies (Table 2). Furthermore, accumulated lactate and pyruvate, not only further substantiate the elevated lactate dehydrogenase levels observed (Table1; Section II.3) but also indicate a possible reduction in oxygen availability in muscles cells, since higher lactate concentrations are observed when exercising under hypoxic conditions (Sumi *et al.*, 2019). Excessive lactate production is neutralised by reacting with bicarbonate (from renal excretion) to produce carbonic acid and eventually carbon dioxide (Salway, 2012). This concurs with the reported reductions in circulating bicarbonate levels after ER (Sumi *et al.*, 2019) and may be associated with post-exercise hyperventilation, impaired glomerular filtration rate or renal function (Section II.3), and metabolic acidosis (Kratz *et al.*, 2002; Salway, 2012; Waśkiewicz *et al.*, 2012). Additionally, elevated lactate concentrations may also be interpreted as an attempt to regenerate oxidised NAD^+ needed in the glycolysis pathway and TCA cycle (Salway, 2012), when an imbalanced redox potential is apparent (White & Schenk, 2012). The latter could also cause the dramatic changes in the TCA cycle intermediates (malate, fumarate, α -ketoglutarate, aconitate, citrate/iso-citrate and

1
2
3 514 succinate) observed by Lewis *et al.* (2010), Nieman *et al.* (2017) and Stander *et al.* (2018). Since the
4
5 515 TCA cycle is the central point of influx for substrate catabolism, increased production of NADH
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7 516 (reduced form of NAD⁺) and flavin adenine nucleotide (FADH₂), is expected due to the immense strain
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9 517 placed on the electron transport chain (ETC) in an attempt to produce sufficient ATP during such
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11 518 activities (Salway, 2012). This, in turn, may result in NADH/FADH₂ accumulation, NAD⁺/FAD
12
13 519 'depletion' and the transient suppression of TCA cycle enzymes (isocitrate dehydrogenase, citrate
14
15 520 synthase and α -ketoglutarate dehydrogenase), in turn causing a slight halt in the cycle flow, and
16
17 521 eventually the accumulation of TCA precursor intermediates (Garrett & Grisham, 2013; Esterhuizen,
18
19 522 van der Westhuizen & Louw, 2017). However, an imbalanced NAD⁺/NADH redox potential after ER
20
21 523 seems to remain controversial, and depend on numerous factors such as exercise duration, intensity and
22
23 524 hydration status (White & Schenk, 2012)
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25
26
27 525 Furthermore, Petibois *et al.* (2002) reported a slight increase in glucose concentrations in 14 athletes
28
29 526 after a 10 km endurance run, which concurred with previous literature (Table 2), and found a positive
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31 527 correlation between capillary blood glucose concentrations and athletic performance. Stander *et al.*
32
33 528 (2018) hypothesised that this may be ascribed to the initial reduction in glycogen and glucose within
34
35 529 90 min after the start of high intensity (>75% VO_{2max}) exercise, possibly resulting in a reduction in
36
37 530 insulin secretion and/or sensitivity (Arakawa *et al.*, 2016; Kim *et al.*, 2007). Kirwan (2010) suggested
38
39 531 that transient inhibition of insulin-dependent components and/or signalling pathways (i.e. glucose
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41 532 transporter type 4, insulin receptor substrate-1 and various kinase) may occur during extensive eccentric
42
43 533 muscle contractions. Since some of these components are vital in terms of glucose absorption into cells,
44
45 534 extracellular accumulation of glucose can be expected and explain the fluctuations in gluconeogenic
46
47 535 intermediates detected after ER (see Table 2 and Section III.3). However, since ER usually extend over
48
49 536 a prolonged period (particularly during marathons and ultra-marathons), athletes would typically ingest
50
51 537 large amounts of carbohydrate-containing foods and beverages, which impact their metabolic profiles,
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53 538 as observed by more robust studies that did not control dietary intake (Jeukendrup, 2011; Pfeiffer *et al.*,
54
55 539 2012; Stander *et al.*, 2018). Waśkiewicz *et al.* (2012) for instance, investigated the acute metabolic
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57 540 adaptations induced by a 24 h ($\pm$ 168.5 km) ultra-marathon race in 14 male athletes. When measuring
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blood glucose levels after the athletes reached marathon distances, non-significant reductions or unchanged glucose concentrations were reported, supporting the results of various other studies (Table 2). However, blood glucose levels started rising at 75 km and were highest after the full ultra-marathon distance, suggesting that these fluctuations may be distance dependent. Suzuki *et al.* (2015) additionally attributed fluctuations in glucose to be dependent on an athlete's individual ability to metabolically adapt to endurance exercise.

(2) Lipid metabolism

Another fuel substrate utilised during high-intensity prolonged exercise, are lipids, that originate from various sources, including adipose tissue, muscle fibres (intra-muscular), cholesterol and dietary triacylglycerols (TAG) (Purdom *et al.*, 2018). Since lipids are considered high-energy content substrates, catabolism thereof is thought to be prominent at higher exercise intensities when glucose and muscle glycogen absorption and catabolism are transiently hampered/insufficient to provide enough energy for the duration of the endurance exercise. Activation of lipid catabolism is mediated by an integrated system of catecholamines including cortisol, growth hormone, glucagon and insulin (inhibition) (Purdom *et al.*, 2018), further substantiating the fluctuations changes in these hormones as reported on numerous occasions (Table 2). Górecka *et al.* (2019) further annotated reduced TAG concentrations, accompanied by elevated glycerol, fatty acids (FA) (Table 2) and angiopoietin-like protein 4, in 10 male athletes after a 100 km ultra-marathon. This profile is supported by various other investigations (Table 2) that strongly indicate increased lipolysis activity with subsequent energy production via β -oxidation and gluconeogenic influx (Salway, 2012). Although lipolysis occurs in the cytoplasm of cells, β -oxidation is confined to the mitochondrial matrix, which is surrounded by a set of membranes that are almost impenetrable for long-chain fatty acids (LCFAs) (Garrett & Grisham, 2013). As such, LCFAs, and in some cases medium-chain fatty acids (activated by acyl-CoA synthetase), are transformed to acyl-carnitines by an outer mitochondrial membrane-associated enzyme known as carnitine palmitoyl transferase (CPT) I, where after an inner membrane-associated translocase facilitates the acyl-carnitine transport into the mitochondria matrix (Salway, 2012; Garrett & Grisham, 2013). Once on the inside of the matrix, CPT II is responsible for the removal of the carnitine

component, and the resulting acyl-CoA (Garrett & Grisham, 2013), is further oxidised by β -oxidation (Salway, 2012). In accordance, Davison *et al.* (2018) used an LC-based serum metabolomics approach to characterise the metabolome changes induced by ER under hypoxic, conditions in 24 male athletes completing a 1h ER session. They observed significantly elevated concentrations of a wide array of carnitines and LCFAs. Although this may merely exploit FA-transport limitations via CPT-1 (Purdom *et al.*, 2018), an alternative explanation may include a saturated/impaired β -oxidation pathway due to the high metabolic strain of high intensity exercise. This is based on the principle that β -oxidation possesses a rate-limiting β -hydroxyacyl dehydrogenase step, consequently giving rise to maximum fat oxidation rates, which have been reported to be optimal at intensities of 45–65% $\text{VO}_{2\text{max}}$ (Purdom *et al.*, 2018). This may be further substantiated by the study of Schader *et al.* (2020), in which higher concentrations of acyl-carnitines and CPT were apparent in less fit/slower athletes (running at lower intensities of 50–60% $\text{VO}_{2\text{max}}$) in comparison to the more fit/faster athletes, thus exploiting the dependence of lipolysis during lower intensity exercise. As such, performing prolonged exercises at intensities exceeding that of the optimal fat oxidation rate could lead to an accumulation of FA or acyl-carnitines, and the subsequent activation of alternative FA acids catabolic pathways. The latter is supported by elevated (peroxisomal and mitochondrial) β -oxidation, ω -oxidation and α -oxidation intermediates observed by numerous other studies (Table 2). This is further depicted by elevated concentrations of various OCFA observed by Stander *et al.* (2018) in 16 marathon athletes, using an untargeted gas chromatography time-of-flight mass spectrometry metabolomics approach. However, OCFA may also originate from cellular membrane autophagy and/or dietary ingestion (Stander *et al.*, 2018; Jenkins *et al.*, 2017).

Up-regulated lipid catabolism directly correlates with increased ketogenesis, since acetyl-CoA is considered the substrate for the latter. Ketones are utilised by the brain and skeletal muscles when glucose/glycogen are significantly reduced (Cahill & Vech, 2003; Meidenbauer, Mukherjee & Seyfried, 2015), as apparent after 60–90 mins after the start of ER. This supports the elevated ketones and ketone-bodies observed after engaging in ER. However, Weibel & Glonek (2007) demonstrated that ketone production may be subjective in endurance athletes, as 9 out of 31 athletes in their study did not appear

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3 595 to produce additional ketones after an ultra-marathon. The researchers attributed this to possible
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5 596 fluctuations in pre-race carbohydrate ingestion and/or the possibility of alternative adaptive metabolic
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7 597 mechanisms in some athletes (Volek *et al.*, 2016). Furthermore, they also hypothesised that the
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9 598 biological medium used (i.e. urine) could have influenced the results (Weibel & Glonek, 2007). It is
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11 599 also important to note that some of the ketone bodies in the studies supporting ketone production after
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13 600 ER (Table 2) are not necessarily products of acetyl-CoA transformation, but rather end-products from
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15 601 branched-chain AA (BCAA) metabolism (discussed in Section III.3).
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19 602 Besides the traditional catabolism of TAG, cholesterol is also considered a viable fuel substrate during
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21 603 ER (Purdom *et al.*, 2018). This lipid-sterol is not only one of the major constituents of cellular
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23 604 membranes, but is also the precursor/substrate for bile salt and steroidogenesis pathways (Salway,
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25 605 2012). As such, elevations in squalene (Stander *et al.*, 2018), cortisol (Arakawa *et al.*, 2016;
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27 606 Mouzopoulos *et al.*, 2007) and other natural steroid-associated metabolites (Daskalaki *et al.*, 2015;
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29 607 Stander *et al.*, 2018; Nieman *et al.*, 2017; Howe *et al.*, 2018) are regularly observed in literature (Table
30
31 608 2). These natural steroid hormones produced from cholesterol (pregnenolone, progesterone, cortisol,
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33 609 etc.) are key components in fuel substrate catabolism mediation as well as energy homeostasis (Garrett
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35 610 & Grisham, 2013; Salway, 2012). Furthermore, Nieman *et al.* (2013) used an untargeted GC-MS and
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37 611 LC-MS metabolomics approach to identify the metabolome adaptations in 15 runners after a three-day
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39 612 training period (2.5 h per day; ± 25.9 km). Reduced concentrations of 22 lysolipid/bile acid metabolites
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41 613 were observed immediately post-exercise, supporting the findings of numerous other studies (Howe *et al.*
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43 614 *et al.*, 2018; Danese *et al.*, 2017). It was hypothesised that these cholesterol-associated alterations may be
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45 615 due to the aforementioned autophagy of cellular membranes, as well as the preferential production of
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47 616 steroid hormones necessary for fuel substrate absorption or catabolism, thus reducing the availability
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49 617 of cholesterol for alternative pathways such as bile production.
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53 618 **(3) AA metabolism**

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55 619 Once strenuous physical activity continues to the point that glycogen stores are ‘depleted’ and FA
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57 620 oxidation has reached a maximal plateau, the metabolism of free AA and muscle protein degradation.
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59 621 This is supported by significant reductions in various AAs, and elevations in AA-associated catabolism
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intermediates (Table 2). Some of these AA intermediates may also be principal components in pathways not directly related to energy production (Salway, 2012; Garrett & Grisham, 2013). In particular, Yamada *et al.* (2016) reported reduced concentrations of tryptophan, accompanied by elevated kynurenine and serotonin in 35 Japanese athletes after completing a 48h ultra-marathon. Although these fluctuations mainly correlated with distance and intensity, kynurenine is also suggested to induce central fatigue, while the increased serotonin (mainly after 45 km) is associated with gluconeogenesis, lipolysis and increased phosphofructokinase activity, which is apparent in athletes (Yamada *et al.*, 2016). However, a reduction in serotonin after running 135 km may be ascribed to an increased ROS production, since the latter inhibits tryptophan hydroxylase, lowering tryptophan and elevating kynurenine production (Yamada *et al.*, 2016), as these are opposing catabolism pathways (Salway, 2012). Real *et al.* (2005), detected elevated concentrations of homocysteine (methionine catabolism intermediate), which is associated with increased risk of cardiovascular dysfunction or malfunction, as indicated by various aforementioned physiologically and immunologically inclined studies (Section II.2). Moreover, numerous studies (Table 2) observed elevated concentrations of *p*-hydroxyphenyllactic acid and *p*-hydroxyphenylacetic acid (phenylalanine and tyrosine intermediates) immediately post-marathon. These metabolites are usually indicative of mild hepatic damage, which correlate with other studies (Section II.3) that reported increased concentrations of hepatic transamination enzymes, including ALT and AST (Table 1). Both of these transamination enzymes are imperative in AA transamination oxidation reactions, which produce aspartate, a precursor of the urea cycle. Further substantiation of increased AA transamination during ER, is indicated by Lewis *et al.* (2010) and Petibois *et al.* (2002), as both identified elevated urea cycle metabolites, including urea, argininosuccinate and creatine, accompanied by reduced levels of ornithine and citrulline, immediately following strenuous activity. Reduced ornithine and citrulline may potentially result from arginase inhibition by N^G-hydroxy-L-arginine, an intermediate produced during elevated nitric oxide synthesis (Morris, 2002). The increased argininosuccinate and creatine concentrations most likely occur as a result of increased AA transaminase activity and the body's need to replenish creatine stores. Creatine is a storable form of ATP (in the form of the aforementioned phosphocreatine) and may spontaneously be converted to creatinine after release of the energy from the high energy phosphate bond (Salway,

650 2012). Therefore, elevated serum creatinine (Table 2) has been associated with increased muscle
651 breakdown and, in combination with the aforementioned elevation in BUN levels (Section II.3), are
652 indicative of a reduced glomerular filtration rate due to renal damage (Salway, 2012; McCullough *et*
653 *al.*, 2011). Traiperm *et al.* (2016) supported this finding in a study aimed at investigating the
654 biochemical effects of marathon running on 50 adolescent athletes (30 males and 20 females). During
655 this investigation, a positive correlation was observed between increased creatinine levels post-
656 marathon and athlete sex, however, no such a correlation could be established with finishing times,
657 indicating that creatinine circulation is comparable across various performance levels (Traiperm *et al.*,
658 2016).

659 In addition, the reduced BCAAs (leucine, isoleucine and valine) and associated α -ketoacids observed
660 by numerous studies (Table 2), indicating increased BCAA oxidation as an alternative fuel substrate
661 (Li *et al.*, 2018). Leucine, among other factors (oxygen, AMP and growth factors), is a key regulator
662 of the mammalian target of rapamycin complex I signalling pathway, which is a natural inhibitor of
663 autophagy (Laplane & Sabatini, 2009). As such, Stander *et al.* (2018) proposed that reduced leucine
664 concentrations may inhibit mammalian target of rapamycin complex I, in turn activating autophagy to
665 release additional membrane-embedded fuel substrates. Leucine also regulates insulin release from
666 pancreatic β -cells when glucose levels become low (Salway, 2012; Kujala *et al.*, 2013), which concurs
667 with the aforementioned transient hampering of insulin secretion. From a neurometabolic perspective,
668 BCAAs are vitally important for the regulation of neurotransmitter (NT) synthesis and transport since
669 catabolism of these are considered nitrogen donors required for excitatory NT (glutamate and γ -
670 aminobutyric acid) production in the brain, the blood-brain transportation of various compounds are
671 highly restricted (Garrett & Grisham, 2013), and BCAAs compete with various NT precursor
672 metabolites for diffusion into the brain (IOM, 2011). Several major classes of NTs have been identified,
673 including cholinergic AA and derivatives of these peptides, and catecholamines (Garrett & Grisham,
674 2013). Of these, Arakawa *et al.* (2016) reported elevated concentrations of the catecholamines
675 including noradrenalin, adrenalin and dopamine in 18 middle-aged athletes completing marathon and

ultra-marathon distances. Since catecholamine production is dependent on various AA precursors, these elevations could also be linked to the aforementioned reductions AA.

(4) Purine metabolism, reactive oxygen species and vitamins

Since the major energy molecules (ATP and guanosine triphosphate [GTP]) utilised in the metabolism are constructed from purine bases (Garrett & Grisham, 2013), it is anticipated that severe energy-requiring states would cause alterations within the purine catabolism pathway. This is depicted by urinary elevations in deoxyinosine, inosine, guanine, xanthosine, and hypoxanthosine by an untargeted LC-MS metabolomics study aimed at investigating whether a 1 h aerobic endurance exercise session would impose metabolic alterations or not (Daskalaki *et al.*, 2015). Additional studies (Zielinski *et al.*, 2009; Lewis *et al.*, 2010) further annotated elevations in AMP, inosine-monophosphate, hypoxanthine, xanthine, urate and allantoin within 60 min after exercise started. Besides ATP and GTP (muscle nucleotide) degradation (Davison *et al.*, 2018), these fluctuations have also been associated with reduced internal oxygen (hypoxia) and elevated ROS (Lewis *et al.*, 2010; Daskalaki *et al.*, 2015; Zielinski *et al.*, 2009; Garrett & Grisham, 2013). One of the many proposed mechanisms by which purine degradation may lead to increased ROS production includes the upregulation of xanthine oxidase (Gomez-Cabrera *et al.*, 2007) during muscle contractions (Steinbacher & Eckl, 2015). This enzyme is responsible for the conversion of hypoxanthine to xanthine and the subsequent production of urate (Garrett & Grisham, 2013; Salway, 2012), thus substantiating the elevated concentrations of urate observed (Table 2). In the event that ROS production exceeds the body's ability to counteract these using anti-oxidants, oxidative stress is increased and may lead to deleterious effects (Ozcan & Ogun, 2015). Mrakic-Sposta *et al.* (2015) investigated elevated ROS, oxidative damage, inflammatory markers (Section II.4) and antioxidant capacity in 46 male athletes before, during, and after a mountain ultra-marathon. Significant elevations in mitochondrial ROS were observed in these athletes after the race, which were associated with excessive ETC leakage due to the tremendous strain placed on the mitochondria for energy production (Mrakic-Sposta *et al.*, 2015). This group also observed elevated levels of 8-hydroxy-2-deoxyguanosine (8-OH-2dG), 8-OH-dG and 8-isoprostane, indicating increased DNA nucleotide oxidation (Turner *et al.*, 2011) and lipid peroxidation due to oxidative stress. Similarly,

Vezzoli *et al.* (2016) investigated the correlation between oxidative stress and the duration of ER in athletes competing in a 50 km (n=6) and 100 km (n=18) ultra-marathon. The authors further annotated significantly elevated oxidised plasma aminothiols (cysteine and cysteinylglycine), oxidised glutathione, and thiobarbituric acid-reactive substances, after the ER events. Considering that elevations in oxidised antioxidants were only deemed statistically significant at 100 km, it may suggest that redox status is dependent on ER duration and intensity. The elevations in thiobarbituric acid-reactive substances, which are reportedly robust markers associated with lipid peroxidation, have been regularly observed (Klapcinska *et al.*, 2013; Oliveira *et al.*, 2017; Howe *et al.*, 2018) following ER and support previous findings of impaired redox status. Furthermore, Mastaloudis *et al.* (2001) observed an increase in vitamin E (tocopherol) clearance in 11 athletes after completion of a 50 km ultra-marathon, due to lipid peroxidation, explaining the reductions in tocopherol-intermediates observed previously (Howe *et al.*, 2018). However, although Traber (2007) indicated a positive correlation between vitamin E supplementation and reduced lipid peroxidation, no such correlation could be established in terms of reduced muscle damage, DNA damage, inflammation and other oxidative stress-associated effects. Moreover, vitamin B5 (pantothenate) was also previously detected in significantly elevated concentrations following ER (Lewis *et al.*, 2010; Lustgarten *et al.*, 2013). Vitamin B5 is one of eight B vitamins which assists in energy production by converting carbohydrates into fuel (i.e. glucose) and is also necessary for the proper functionality of the nervous system, and utilisation of fats and proteins for energy.

IV. In a nutshell, what does this mean?

(1) The problem and its roots:

Despite previous attempts to elucidate the systematic biochemical effects of ER (listed in Tables 1 and 2), a definite conclusion whether it is overall more deleterious or beneficial to human health remains unresolved. This may largely be ascribed to the fact that no human-based study can compensate for the various inevitable phenotypic and genotypic factors. In synopsis of the reviewed studies, blood parameters were highly subjective to hydration status (i.e. overhydration and dehydration), whereas cardiovascular, muscle, renal, hepatic, and structural (bone) alterations, and subsequent immune

responses, were mostly dependent on the duration and intensity of an acute bout of ER, age, sex, and the fitness status of athletes. Metabolically, dietary regimens or preferences (both during and pre-exercise), along with an athlete's ability to adapt to vigorous metabolic switching, seems to add to these the various controversies among research groups. In terms of athlete sex correlations, is clear from Tables 1 and 2 that very few studies included female athletes, which may be attributed to the increased susceptibility of female athletes to hormonal indifferences, cardiovascular diseases, and a higher probability of female triad syndrome in more experienced athletes. Female triad syndrome is associated with reduced energy availability and bone mineral density, accompanied by menstrual dysfunction (Daily & Stumbo, 2018). Nevertheless, considering that female athlete participation in endurance races increases exponentially per annum, future research should include more female participants in cohorts. Additionally, less frequently described variation factors, such as (1) fitness level (not based on experience), (2) detailed notation of recovery period since the last ER event or training session, (3) the effects of the short resting periods or a reduction in exercise intensity during repeated bouts, and (4) participant psychological rationale (competitive race determinants vs non-competitive treadmill exercise) should also be taken into account.

(2) A possible proficiency classification system

Considering that athletic proficiency and exercise intensity/duration are some of the most influential factors determining biomarker adaptations during and after ER, adequate standards for a uniform classification are of utmost importance. Although Stoggl & Sperlich (2015) provided a clear structure for classifying exercise intensity, to our knowledge, no such standard is available for classifying ER athletes according to investigative distance-specific proficiency. As such, we propose a structured proficiency classification system (Table 3), collectively based on the literature presented in Tables 1 and 2. Classification should firstly be based on the investigative distance of the specific experimental protocol. Hereafter, proficiency can be assigned based on two of the following three factors, either individually or when taking the average of the entire cohort into account: (1) total/average 'years of experience' in terms of participation or training, (2) the (average) 'number of competitive endurance races' to date, and/or (3) 'weekly training km' (average). For example, when investigating the effects

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757 of a marathon in athletes with four years of experience, who have competed in an average of five
758 endurance races, and whom train by running an average of 35 km weekly, the following classification
759 would be made based on Table 3: A marathon would be classified as ‘level 3’, and based on
760 ‘experience’ and/or ‘number of competitive endurance races’ and/or ‘weekly training’ (any two of these
761 factors), these athletes would be classified as ‘intermediate athletes’. In the event that two of the three
762 criteria per investigative distance are not applicable to the athletes (either individually or when
763 considering the cohort average), we suggest basing proficiency on the average ‘weekly training km’ of
764 athletes only, thus avoiding bias towards competitive vs non-competitive athletes.

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Table 3: Proposed proficiency classification system for endurance running investigations.

Investigative distance level	Novice athletes (0≤2yrs experience)		Intermediate athletes (3<5yrs experience)		Master athletes (5<10yrs experience)		Elite athletes (≥10yrs experience)	
	Number of competitive endurance races	Weekly training (km)	Number of competitive endurance races	Weekly training (km)	Number of competitive endurance races	Weekly training (km)	Number of competitive endurance races	Weekly training (km)
Level 1 (0<15 km)	0–2	0.0–3.5	3–5	3.5<7.0	5–10	7.0<10.5	10≤	≥10.5
Level 2 (15<30 km)		<18.5		18.5<22.0		22.0<25.5		≥25.5
Level 3 (30<50 km)		<35.0		35.0<40.0		40.0<45.0		≥45.0
Level 4 (50<80 km)		<57.5		57.5<65.0		65.0<72.5		≥72.5
Level 5 (80<150 km)		<87.5		87.5<95.0		95.0<102.5		≥102.5
Level 6 (150<300 km)		<180.7		180.7<225.0		225.0<262.5		≥262.5
Level 7 (300 km≤)		<350.0		350.0<400.0		400.0<450.0		≥450.0

V. Conclusion

(1) To date, various studies established a link between an athlete's hydration status and anaemia, as well as an electrolyte imbalance.

(2) Furthermore, cardiovascular markers indicate an amplified workload mainly due to increased volume or pressure and wall stretching, however, these markers cannot necessarily be associated with dysfunction and damage. Studies have also indicated myocardial protection with possible increased antioxidant capacity in those who exercise or compete regularly.

(3) On the other hand, muscle, renal and hepatic markers indicate a cascade of events ultimately leading to substantial chronic inflammation due to mechanical shearing and ROS-related damage.

(4) Some studies further indicate transient renal and hepatic injury or failure; however, these detrimental effects have been correlated with age (due to reduced adaptive abilities), sex, diet, genetic factors, as well as the intensity, duration and frequency of exercise.

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(5) Additionally, metabolomics studies generally indicate the substantial fuel substrate adaptations due to an increased metabolic fuel dependence during ER, without truly identifying specific beneficial or detrimental effects. Hence, the perceived health-promoting or detrimental effects of ER can be seen on a physiological, immunological, and metabolic level.

(6) Unfortunately, limited research has relied on integrated scientific approaches which would show the overall systemic effects of induced by ER. Considering this, future research approaches may benefit from a systems biology perspective, accounting for athlete sex, age and fitness levels.

(7) Such information may provide a better understanding of the various biological and biochemical aspects of ER, giving clues to better, or even individualised, (a) athletic performance optimisation methods, (b) systemic damage prevention strategies, and (c) optimised recovery methods, which may be profitable going forward.

VI. Administrative information

(1) Author contribution

Mrs Zinandré Stander: Conceptualisation, writing – original draft preparation, writing – review and editing; **Dr Laneke Luies:** Conceptualisation, supervision, writing – review and editing; **Prof Du Toit Loots:** Conceptualisation, supervision, writing – review and editing

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(3) Conflict/declarations of interest

The authors declare that there are no conflicts/declarations of interest and that this manuscript, and the work described therein, is unpublished and has not been submitted for publication elsewhere.

(4) Compliance with ethical requirements

No ethical approval was required.

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VIII. Appendix A

(1) Abbreviations

8-OH-dG: 8-Hydroxy-2-deoxyguanosine; AA: Amino acids; AGE: Advance glycation end-products; ALT: Alanine transferase; AMP: Adenosine monophosphate; AST: Aspartate transferase; ATP: Adenosine triphosphate; BCAA: Branched-chain amino acid; BNP: Brain natriuretic peptide; BUN: Blood-urea-nitrogen; Ca^{2+} : Calcium; ccCK: Caspase-cleaved cytokeratin; CK-MB: Creatine kinase-myocardial band; Cl⁻: Chloride; Co-A: Coenzyme A; COMP: Cartilage oligomeric matrix protein; CPT: Carnitine palmitoyl transferase; CRP: C-reactive protein; cTnI: Cardiac troponin I; cTnT: Cardiac troponin T; CXT: Cross-linked C-telopeptide of type I collagen; DNA: Deoxyribonucleic acid; EIH: Exercise-induced hypertension; ER: Endurance running; esRAGE: Endogenous secretory receptor of advance glycation end-products; ETC: Electron transport chain; FA: Fatty acids; FAD^{+} : Flavin adenine nucleotide; GC: Gas chromatography; GTP: Guanosine triphosphate; Hb: Haemoglobin; Hct: Hematocrit; H-FABP: Heart-type fatty acid-binding protein; HSP: Heat shock protein; $\text{IFN-}\gamma$: Interferon-gamma; Ig: Immunoglobulin; IL: Interleukin; IMA: Ischemia-modified albumin; K^{+} : Potassium; LC: Liquid chromatography; LCFA: Long chain fatty acids; Mb: Myoglobin; MCH: Mean corpuscular haemoglobin volume; MCHC: Mean corpus haemoglobin concentration; MCV: Mean corpus volume; Mg^{2+} : Magnesium; MMP-3: Metalloproteinase-3; MS: Mass spectrometry; Na^{+} : Sodium; NAD^{+} : Nicotinamide adenine dinucleotide; NT: Neurotransmitter ; NT-proBNP: N-terminal pro-brain natriuretic peptide; OWE: Open window effect; PINP: Pro-collagen type I N-terminal pro-peptide; P^{3-} : Phosphate; PL: Platelets; RAGE: Receptor of advance glycation end-products; RANKL: Receptor activator of nuclear factor κB ligand; RBC: Red blood cell; ROS: Reactive oxygen species; TAG: Triacylglycerol; TBARS: Thiobarbituric acid reactive substances; TCA: Tricarboxylic acid; TIM: Tissue injury model; $\text{TNF-}\alpha$: Tumour necrosis factor alpha; TP: Total protein; $\text{VO}_{2\text{max}}$: Maximum volume of oxygen; WBC: White blood cells/leukocytes; W_{max} : Maximal work capacity; YKL-40: Chitinase 3-like protein.

Table 1: Endurance running-induced physiological and immunological-associated biomarker adaptations reported in recent (2000-2020) literature (arranged according to publication year).

Reference	Exercise type	Cohort	Sampling media	Physiological and immunological adaptations when compared to baseline measurements	
Kratz, et al. [49]	Marathon	37 participants (32 male; 5 female)	Blood; before, within 4 h after and 24 h ⁺ post-race	Within 4 h post-race: Blood biochemistry: RBC (↓) ^a ; Hct (↓) ^a ; Hb (↑) ^a ; PL (↑) ^a ; Alb (↑) ^a ; TP (↑) ^a ; MCV (↓) ^a ; MCH (↑) ^a ; MCHC (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; ALT (↑) ^a ; AST (↑) ^a ; Bilirubin (↑) ^a , ALP (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; cTnI (↑) ^a ; Mb (↑) ^a Renal damage: BUN (↑) ^a Electrolyte markers: Na ⁺ (↓)*; K ⁺ (↓)*; Cl ⁻ (↓) ^a ; Mg ²⁺ (↓) ^a ; Ca ²⁺ (↑) ^a ; P ³⁻ (↑) ^a Immune response: WBC (↑) ^a ; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Basophils (↑) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ; Globulin (↓) ^a	
Niessner, et al. [40]	Marathon	19 participants (17 male; 2 female)	Blood; before and immediately after the race	Post-race: Cardiac muscle damage: Pro-ANP (↑) ^a ; NT-proBNP (↑)* Hormonal response: Aldosterone (↑) ^a	
Uchakin, et al. [50]	Marathon (White Rock and Cowton marathons)	15 male participants	Blood; before, 20 min and 60 min after the race as well as 2 ⁺ , 5 ⁺ and 8-days ⁺ post-race	Within 20 min post-race: Blood biochemistry: Hct (↑)*; Hb (↑)* Muscle and hepatic damage: CK (↑) ^a Immune response: Granulocytes (↑) ^a ; IL-6 (↑) ^a ; TNF-α (↑) ^a ; Lymphocytes (↓)*; IL-1β (-) LPS stimulated: IL-2 (↓) ^a ; IL-1β (↓)*; IFN-γ (↓) ^a ; IL-10 (↓)*; TNF-α (↓) ^a ; IL-6 (↓)* Hormonal response: ACTH (↑) ^a ; β-endorphin (↑) ^a ; Growth hormone (↑) ^a ; Cortisol (↑) ^a	Within 60 min post-race: Blood biochemistry: Hct (↑)*, ^c ; Hb (↑)*, ^c Muscle and hepatic damage: CK (↑) ^{a,b} Immune response: Granulocytes (↓) ^{a,c} ; IL-6 (↑) ^{a,c} ; TNF-α (↑) ^a ; Lymphocytes (↓) ^{a,c} ; IL-1β (-) LPS stimulated: IL-2 (↓) ^{a,c} ; IL-1β (↓)*, ^b ; IFN-γ (↓) ^{a,c} ; IL-10 (↓)*, ^b ; TNF-α (↓) ^{a,b} ; IL-6 (↓)*, ^b Hormonal response: ACTH (↑) ^{a,c} ; β-endorphin (↑) ^{a,c} ; Growth hormone (↑) ^{a,c} ; Cortisol (↑) ^{a,c}
Goudie, et al. [44]	Marathon	14 participants (genders not provided)	Blood; patients admitted to St Thomas hospital post-race	Post-race: Electrolyte markers: Na ⁺ (↓) ^a	
Kratz, et al. [24]	Marathon	32 participants (27 male; 5 female)	Blood; before and immediately after the race	Post-race: Blood biochemistry: RBC (↓) ^a ; Hct (↑) ^a ; Hb (↑) ^a ; PL (↑)*; MCV (↓) ^a ; MCH (↑)*; MCHC (↑) ^a , Reticulocyte (↑) ^a Immune response: WBC (↑) ^a ; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Basophils (↓) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ;	
Leers, et al. [51]	Marathon	27 participants (25 male; 2 female)	Blood; before, immediately after and 24 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnT (↑) ^a ; BNP (↑)*; NT-proBNP (↑) ^a Electrolyte markers: Na ⁺ (↓)*; Cl ⁻ (↓) ^a ; K ⁺ (-)	
Kim, et al. [52]	Ultra-marathon (200 km)	54 male participants	Blood; before, after 100 km and	100 km of race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a	Post-race: Muscle and hepatic damage: CK (↑) ^{a,d} ; LDH (↑) ^{a,d} ; AST (↑) ^{a,d} ; ALT (↑) ^a

			immediately post-race	Immune response: Hs-CRP ($\uparrow$) ^a ; IL-6 ($\uparrow$) ^a ; TNF- α ($\uparrow$) [*] Cartilage damage: COMP ($\uparrow$) ^a Blood biochemistry: Hct ($\uparrow$) [*]	Immune response: Hs-CRP ($\uparrow$) ^{a,d} ; IL-6 ($\uparrow$) ^{a,d} ; TNF- α ($\uparrow$) [*] Cartilage damage: COMP ($\uparrow$) ^{a,d} Blood biochemistry: Hct ($\uparrow$) ^{*,b}
Mouzopoulos, et al. [53]	Ultra-marathon (245 km)	16 male participants	Blood; 5-days before, immediately after as well as 1 ⁺ , 3 ⁺ and 5-days ⁺ post-race	Post-race: Bone biochemistry: ICTP ($\uparrow$) [*] ; PICP ($\downarrow$) ^a ; Osteocalcin ($\downarrow$) ^a ; ALP ($\downarrow$) ^a Hormonal response: Parathyroid hormone ($\uparrow$) ^a Electrolyte markers: Ca ⁺ ($\downarrow$) [*]	
Siegel, et al. [39]	Marathon	33 participants (<i>sex not provided</i>)	Blood; before and after the race	Post-race: Immune response: IL-6 ($\uparrow$) ^a ; CRP (–) Muscle and hepatic damage: CK ($\uparrow$) ^a Hormonal response: Prolactin ($\uparrow$) ^a ; Arginine vasopressin ($\uparrow$) ^a Renal damage: BUN ($\uparrow$) ^a	
Lippi, et al. [54]	Half-marathon	15 male participants	Blood; before, immediately after, 3 h, ++	Post-race: Muscle and hepatic damage: CK ($\uparrow$) ^a ; LDH ($\uparrow$) ^a ; AST ($\uparrow$) ^a Cardiac muscle damage: CK-MB ($\uparrow$) ^a ; Mb ($\uparrow$) ^a ; cTnT (–)	
Knechtle, et al. [36]	Ultra-marathon (100 km)	39 male participants	Blood and urine; before and immediately after	Post-race: Blood biochemistry: Hct ($\downarrow$) ^a Electrolyte markers: Na ⁺ ($\downarrow$) ^a	
Kersch-Schindl, et al. [55]	Ultra-marathon (246 km)	18 participants (16 male; 2 female)	Blood; before, within 15 min after as well as 3-days ⁺ post-race	Post-race: Bone biochemistry: CTX ($\uparrow$) ^a , Osteocalcin ($\downarrow$) ^a , Osteoprotegerin ($\uparrow$) ^a , RANKL ($\uparrow$) [*]	
Fu, et al. [56]	Half-marathon	17 adolescent male participants	Blood; before, within 12 min after as well as 4 h ⁺ post-race	Post-race: Cardiac muscle damage: cTnI ($\uparrow$) ^a , NT-proBNP ($\uparrow$) ^a	
Burge, et al. [38]	Ultra-marathon (100 km)	50 male participants	Blood and urine; before and after the race	Post-race: Blood biochemistry: Hct ($\downarrow$) [*] ; Hb ($\downarrow$) [*] Electrolyte markers: Na ⁺ ($\uparrow$) ^a , K ⁺ ($\uparrow$) ^a Hormonal response: Aldosterone ($\uparrow$) ^a , Copeptin ($\uparrow$) ^a	
Kipps, et al. [42]	Marathon	88 participants (69 male; 42 female)	Blood; before and after the race	Post-race: Electrolyte markers: Na ⁺ ($\downarrow$) ^a	
Knechtle, et al. [41]	Ultra-marathon (100 km)	145 male participants	Blood; before and immediately after the race	Post-race: Blood biochemistry: Hct ($\downarrow$) ^a ; Hb ($\downarrow$) ^a Electrolyte markers: Na ⁺ ($\downarrow$) [*]	
Lippi, et al. [57]	Half-marathon	15 male participants	Blood; pre-fasting, 48 h after last training session,	Post-race: Muscle and hepatic damage: CK ($\uparrow$) ^a ; LDH ($\uparrow$) ^a ; AST ($\uparrow$) ^a ; GGT ($\uparrow$) ^a ; Bilirubin ($\uparrow$) ^a ; ALT (–); ALP (–)	

			immediately after the race as well as 3 h ⁺ , 6 h ⁺ and 24 h ⁺ post-race			
McCullough, et al. [58]	Marathon	25 participants (12 male; 13 female)	Blood: before, after and 24 h ⁺ post-race	Within 4 h post-race: Blood biochemistry: Alb (↑) ^a ; TP (↑) ^a ; MCV (↓) ^a ; MCHC (↑) ^a ; MCH (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; ALT (↑) ^a ; AST (↑) ^a ; Bilirubin (↑) ^a ; ALP (↑) ^a ; Aldolase (↑) ^a , ALP (↑) ^a Cardiac muscle damage: CK-MB (↑) ^a ; cTnI (↑) ^a ; BNP (↑) ^a Renal damage: BUN (↑) ^a ; Cystatin (↑) [*] ; NGAL (↑) ^a ; KIM-1 (↑) ^a Electrolyte markers: Na ⁺ (↑) [*] ; K ⁺ (↑) ^a Immune response: Globulin (–)		
La Gerche, et al. [59]	Marathon	9 cyclists ⁺ , 13 triathlon- ⁺ and 7 marathon participants	Blood; 2–3 weeks before, immediately after and 6–11 days ⁺ post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; BNP (↑) ^a (<i>correlated with cardio-graphic tests</i>) ⁺		
Waśkiewicz, et al. [60]	Ultra-marathon (±168.5 km; 24 h)	19 male participants	Blood; before, at marathon distance, within 12 h and immediately after the ultra-marathon	Marathon length: Blood biochemistry: RBC (↑) [*] ; Hb (↑) [*] ; Hct (↑) [*] ; PL (↑) [*] ; MCV (↓) [*] ; CO ₂ (↓) [*] ; O ₂ (↓) [*] Muscle and hepatic damage: CK (↑) ^a ; AST (–); ALT (–); GGT (–) Electrolyte markers: Na ⁺ (↑) [*] ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) [*] Immune response: WBC (↑) ^a ; Lymphocytes (↑) [*] ; Monocytes (↑) ^a ; Neutrophils (↑) ^a ; Eosinophils (↓) [*] ; Basophils (↑) [*] ; IL-6 (↑) ^a , CRP (–);	12 h of running: Blood biochemistry: RBC (↑) ^{*,c} ; Hb (↑) ^{*,c} ; PL (↑) ^{*,c} ; O ₂ (↑) ^{*,b} ; Hct (↓) ^{*,c} ; MCV (↓) [*] ; CO ₂ (↓) ^{a,c} Muscle and hepatic damage: CK (↑) ^{a,b} ; AST (–); ALT (–); GGT (–) Electrolyte markers: Na ⁺ (↑) ^{*,b} ; K ⁺ (↑) ^{a,b} ; Ca ²⁺ (↓) ^a Immune response: WBC (↑) ^{a,c} ; Lymphocytes (↓) ^{*,c} ; Monocytes (↑) ^{a,b} ; Neutrophils (↑) ^{a,b} ; Eosinophils (↓) ^{a,c} ; Basophils (↑) ^{*,c} ; IL-6 (↑) ^{a,b} , CRP (↑) ^a	Post-race: Blood biochemistry: RBC (↑) ^{*,c} ; Hb (↑) ^{*,c} ; PL (↑) ^{*,c} ; O ₂ (↑) ^{a,b} ; Hct (↓) ^{*,c} ; MCV (↓) [*] ; CO ₂ (↓) ^{a,c} Muscle and hepatic damage: CK (↑) ^{a,b} ; AST (↑) ^a ; ALT (↑) ^a ; GGT (↑) [*] Electrolyte markers: Na ⁺ (↓) ^{*,c} ; K ⁺ (↑) ^{a,b} ; Ca ²⁺ (↑) ^{*,b} Immune response: WBC (↑) ^{a,b} ; Lymphocytes (↓) ^{*,b} ; Monocytes (↑) ^{a,b} ; Neutrophils (↑) ^{a,c} ; Eosinophils (↓) ^{a,b} ; Basophils (↑) ^{a,-} ; IL-6 (↑) ^{a,b} , CRP (↑) ^{a,b}
Costa, et al. [45]	Multi-stage-marathon (225 km)	74 participants (46 male; 28 female) and 12 controls ⁺ (5 male; 7 female)	Blood; before and after each stage	Stages 1-5: Electrolyte markers: Na ⁺ (↓) [*] (<i>hyponatremia in eight athletes</i>)		
Klapcinska, et al. [61]	Ultra-marathon (48 h; 183–320 km)	7 male participants	Blood; before, after running 12 h, 24 h and within 10 min after the race as well as 24 h ⁺ and 28 h ⁺ post-race	12 h of running: Blood biochemistry: RBC (↑) [*] ; Hct (–); Hb (↑) [*] ; PL (↑) ^a ; MCV (↓) ^a ; MCH (↑) [*] ; MCHC (↑) ^a ; pCO ₂ (↓) ^a Electrolyte markers: Na ⁺ (↓) [*] ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) [*]	24 h of running: Blood biochemistry: RBC (–) ^c ; Hct (↓) ^{*,c} ; Hb (↑) ^{*,c} ; PL (↑) ^{a,c} ; MCV (↓) ^{a,c} ; MCH (↑) [*] ; MCHC (↑) ^{a,b} pCO ₂ (↓) ^{a,b} Electrolyte markers: Na ⁺ (↓) ^{*,c} ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) ^{a,c}	Post-race: Blood biochemistry: RBC (↓) [*] ; Hct (↓) ^a ; Hb (↑) [*] ; PL (↑) ^{*,c} ; MCV (↓) ^{a,b} ; MCH (↑) [*] ; MCHC (↑) ^{a,c} ; pCO ₂ (↓) ^{a,c} Electrolyte markers: Na ⁺ (↓) ^{a,c} ; K ⁺ (↑) ^a ; Ca ²⁺ (↓) ^{a,c}

				Iron panel: Ferritin (↑) ^a ; Iron (↓)* Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; Lymphocytes (↑)*	Iron panel: Ferritin (↑) ^{a,b} ; Iron (↓)* ^b Immune response: WBC (↑) ^{a,c} ; Neutrophils (↑) ^{a,c} ; Monocytes (↑) ^{a,c} ; Lymphocytes (↑)*	Iron panel: Ferritin (↑) ^{a,b} ; Iron (↓)* ^c Immune response: WBC (↑) ^{a,c} ; Neutrophils (↑) ^{a,c} ; Monocytes (↑) ^{a,c} ; Lymphocytes (↑)* ^c	
Robach, et al. [31]	Ultra-marathon (166 km)	22 male participants	Blood, before and within 20 min after the race as well as 2 ⁺ , 5 ⁺ , 9 ⁺ and 16 ⁺ – days post-race	Post-race: Blood biochemistry: Hb (↑)*, Haptoglobin (↓) ^a Cardiac muscle damage: CK-MB (↑) ^a ; Mb (↑) ^a ; cTnT (–) Muscle and hepatic damage: LDH (↑) ^a ; Bilirubin (↑)* Immune response: WBC (↑) ^a , CRP (↑) ^a , Orosomucoid (↑) ^a			
Salvagno, et al. [62]	Ultra-marathon (60 km)	18 participants (<i>sex not provided</i>)	Blood; before, within 10 min after as well as 1 h ⁺ after	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; NT-proBNP (↑) ^a ; Galectin-3 (↑) ^a			
Vuolteenaho, et al. [63]	Marathon	46 male participants	Blood; before and immediately after	Post-race: Cartilage damage: COMP (–); MMP (↑) ^a ; YKL (↑) ^a Hormonal response: Adiponectin (↑) ^a ; Leptin (↓)*; Resistin (↑) ^a			
Hewing, et al. [64]	Marathon	167 participants (78 male; 89 female)	Blood; before, immediately after as well as 2 weeks ⁺ post-race	Post-race: Blood biochemistry: Hb (↑) ^a ; Hct (↑) ^a ; TP (↑) ^a Electrolyte markers: Na ⁺ (↑) ^a Cardiac muscle damage: cTnT (↑) ^a ; NT-proBNP (↑) ^a Renal damage: Cystatin C (↑) ^a Immune response: CRP (↓) ^a			
Jastrzebski, et al. [65]-	Ultra-marathon (100 km)	14 male participants	Blood; before, after 25 km, 50 km, 75 km as well as immediately after the 100 km and 24 h ⁺ post-race	After 25 km: Muscle and hepatic damage: CK (↑) ^a , LDH (↑) ^a , AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a .	After 50 km: Muscle and hepatic damage: CK (↑) ^a , LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a .	After 75 km: Muscle and hepatic damage: CK (↑) ^a , LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a .	After 100 km: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a Immune response: CRP (↑) ^a .
Khodae, et al. [66]	Ultra-marathon (161 km)	20 pre-marathon participants (14 male; 6 female) and 21 post-marathon participants (17 male; 4 female)	Blood; before and immediately after	Post-race: Blood biochemistry: Hct (↓)*; Hb (↓)* Electrolyte markers: Na ⁺ (↓) ^a ; Cl [–] (↓) ^a ; K ⁺ (–) Renal damage: BUN (↑) ^a Cardiac muscle damage: cTnI (↑) ^a			
Kersch-Schindl, et al. [67]	Ultra-marathon (246 km)	19 participants (18 male; 1 female)	Blood; before, within 15 min after as well as 3-days ⁺ post-race	Post-race: Bone biochemistry: CTX (↑) ^a , Sclerostin (↓)*, Dkk-1 (↓) ^a , PINP (↓) ^a , Cathepsin K (↓)* Muscle and hepatic damage: Myostatin (↑) ^a , Follistatin (↑) ^a			

Li, et al. [68]	5 km race	18 participants (8 male; 8 female)	Saliva; before and 10 min after the race	Post-race: Salivary defense (<i>sex dependent</i>): IgA (↑) ^a ; α-amylase (↑) ^a , TP (↑) ^a , lactoferrin (↑)*			
Suzuki, et al. [69]	5 h running exercise (Participant A= 34.14 km; participant B=43.05 km)	2 female participants	Blood; before, within 60 min after as well as 1 ⁺ , 3 and 5 h post-race	Participant A post-race: Blood biochemistry: Alb (↓) Hormonal response: Insulin (↑); Glucagon (↑); ACTH (↑); Vasopressin (↑)		Participant B post-race: Blood biochemistry: Alb (↑) Hormonal response: Insulin (↓); Glucagon (↑); ACTH (↑); Vasopressin (↑)	
Arakawa, et al. [70]	Ultra-marathon (130 km)	18 middle-aged male participants	Blood; before, after 42 km (marathon), immediately after as well as 3 ⁺ , 5 ⁺ and 7 days ⁺ post-race	Marathon length: Blood biochemistry: RBC (↑)*; Hct (↑)*; Hb (↑)*; TP (↑) ^a ; Alb (↑) ^a ; MCV (↓)*; MCH (↑)*; MCHC (↑)* Muscle and hepatic damage: CK (↑)*; LDH (↑) ^a ; AST (↑)*; ALT (↑)*; GGT (↑)*; Bilirubin (↑)* Renal damage: BUN (↑)* Hormonal response: Insulin (↓) ^a ; Leptin (↑)*; Adiponectin (↑)* Immune response: WBC (↑) ^a ; IL-6 (↑) ^a ; CRP (–); TNF-α (↑)* Iron panel: Ferritin (↑)*		Post-race: Blood biochemistry: RBC (↑)* ^c ; Hct (↑)* ^c ; Hb (↑)* ^c ; TP (↑)* ^c ; Alb (↑) ^{a,c} ; MCHC (↑)* ^c ; MCV (↓) ^{a,c} ; MCH (↓)* ^c Muscle and hepatic damage: CK (↑) ^{a,b} ; LDH (↑) ^{a,b} ; AST (↑) ^{a,b} ; ALT (↑) ^{a,b} ; GGT (↑)* ^b ; Bilirubin (↑) ^{a,b} Renal damage: BUN (↑) ^{a,b} Hormonal response: Insulin (↓) ^{a,c} ; Leptin (↓) ^{a,c} ; Adiponectin (↑)* ^b Immune response: WBC (↑) ^{a,b} ; IL-6 (↑) ^{a,c,*} ; CRP (↑) ^{a,b} ; TNF-α (↓)* ^c Iron panel: Ferritin (↑) ^{a,b} ;	
Bekos, et al. [71]	Marathon and half-marathon	34 marathon participants (24 male; 10 female), 36 half-marathon participants (19 male; 17 female) and 30 sedentary controls ⁺ (17 male; 13 female)	Blood and urine; before and immediately after as well as 2–7 days ⁺ post-race	Half-marathon: Blood biochemistry: RBC (↑) ^a ; Hb (↑) ^a ; PL (↑) ^a ; MCV (↑) ^a ; MCH (↑) ^a ; MCHC (↑) ^a ; Hct (–) Muscle and hepatic damage: CK (–); GGT (–) Cardiac muscle damage: CK-MB (–); sST2 (–) Electrolyte markers: Serum Na ⁺ (↑) ^a Urine fraction Na ⁺ (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; RAGE (↑) ^a ; AGE-CML (↑) ^a ; Lymphocytes (↓) ^a ; Monocytes (↓) ^a ; CRP (–); esRAGE (–); IL-1RA (–); IL-33 (–) Apoptosis: Serum HMGB1 (↑) ^a ; Serum cytokeratin 18 (↑) ^a ; Urine cytokeratin 18 (↑) ^a		Marathon: Blood biochemistry: RBC (↑) ^a ; PL (↑) ^a ; MCV (↑) ^a ; MCH (↑) ^a ; MCHC (↑) ^a ; Hct (–); Hb (–) Muscle and hepatic damage: CK (↑) ^a ; GGT (–) Cardiac muscle damage: CK-MB (↑) ^a ; sST2 (↑) ^a Electrolyte markers: Serum Na ⁺ (↑) ^a Urine fraction Na ⁺ (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; AGE-CML (↑) ^{a,d} ; Lymphocytes (↓) ^a ; Monocytes (–); CRP (–); RAGE (–); esRAGE (–); IL-1 (–); IL-33 (–) Apoptosis: Serum HMGB1 (↑) ^a ; Serum cytokeratin 18 (↑); Urine cytokeratin 18 (↑)*	
Jastrzebski, et al. [72]	Ultra-marathon (100 km)	14 male participants	Blood; before, after 20 km, 50 km, 75 km, and immediately after and 24 h ⁺ post-race	25 km of race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)*; MCHC (↓)*	50 km of race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; MCHC (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)*	75 km of race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)*; MCHC (↓)*	Post-race: Blood cells: Hct (↑)*; Hb (↑)*; PL (↑)*; MCHC (↑)*; RBC (↓)*; MCV (↓)*; MCH (↓)*

				Electrolytes: Na ⁺ (↓)*; Ca ²⁺ (↓)*; Cl ⁻ (↓)*; K ⁺ (↑)*. Cardiac muscle damage: Mb (↑)*. Muscle and hepatic damage: Bilirubin (↑)*. Immune response: WBC (↑)*	Electrolytes: Na ⁺ (↓)*; Ca ²⁺ (↓)*; Cl ⁻ (↑)*; K ⁺ (↑)*. Cardiac muscle damage: Mb (↑) ^a . Muscle and hepatic damage: Bilirubin (↑)* Immune response: WBC (↑)*	Electrolytes: Na ⁺ (↓)*; Ca ²⁺ (↓)* ; Cl ⁻ (↓)*; K ⁺ (↑)* Cardiac muscle damage: Mb (↑) ^a . Muscle and hepatic damage: Bilirubin (↑)* Immune response: WBC (↑)	Electrolytes: Na ⁺ (↑)*; K ⁺ (↑)*; Ca ²⁺ (↓)* ; Cl ⁻ (↓)* Cardiac muscle damage: Mb (↑) ^a . Muscle and hepatic damage: Bilirubin (↑) ^a . Immune response: WBC (↑)*
Nielsen, et al. [73]	Marathon and half-marathon	14 male marathon participants, 16 half-marathon participants (8 male; 8 female) and 10 male ranger cadets ⁺	Blood; before and immediately after each race	Half-marathon length: Blood biochemistry: Hct (–); Hb (–); PL (↑) ^a Immune response: WBC (↑) ^a ; IL-1β (↑)*; IL-6 (↑) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF-α (↓)*; IL-2 (–); IL-4 (–); IL-5 (–); IL-12 (–); IFN-γ (–); GM-CFS (–); VEGF (–) LPS stimulated: IL-1β (↓) ^a ; IL-6 (↓) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF (↓) ^a		Marathon length: Blood biochemistry: Hct (–); Hb (–); PL (↑) ^a Immune response: WBC (↑); IL-6 (↑) ^a ; IL-8 (↑) ^a ; IL-10 (↑) ^a ; TNF-α (↑)*; IL-1β (↓)*; IL-4 (↓)*; IL-12 (↓)*; VEGF (↓)*; IL-2 (–); IL-5 (–); IFN-γ (–); GM-CFS (–) No LPS stimulation of cytokines	
Shin, et al. [74]	Ultra-marathon (308 km)	17 marathon participants, 17 ultra-marathon (100 km) participants and 17 ultra-marathon participants (308 km) (<i>sex not provided</i>)	Blood; before, at marathon distance, after 100 km and immediately post-race	Marathon distance: Blood biochemistry: TP (↑) ^a ; Alb (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑)*; GGT (↑) ^a , ALP (↑) ^a Renal damage: BUN (↑) ^a	After 100 km: Blood biochemistry: TP (↑)*; Alb (↑)* Muscle and hepatic damage: CK (↑) ^{a,d} ; LDH (↑) ^{a,d} ; AST (↑) ^{a,d} ; ALT (↑) ^{a,d} ; Bilirubin (↑) ^a ; GGT (↓)*, ALP (↑)* Renal damage: BUN (↑) ^{a,d}	Post-race: Blood biochemistry: TP (↑) ^a , Alb (↓) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑) ^a ; GGT (↑) ^{a,d} , ALP (↓)* Renal damage: BUN (↑) ^{a,d}	
Danese, et al. [75]	Half-marathon	30 participants (21 male; 9 female)	Blood; before and immediately after the race	Post-race: Blood biochemistry: Hct (–); Hb (–)			
Mündermann, et al. [76]	Marathon	45 male participants	Blood; before, after a 10-week training period ⁺ , immediately after the marathon as well as 24 h ⁺ post-race	Post-race: Cartilage damage: COMP (↑) ^a Immune response: Hs-CRP (↓) ^a , TNF-α (↓) ^a , IL-6 (↑) ^a			
Oliveira, et al. [4]	Marathon (hot and temperate temperatures)	26 male participants	Blood and urine, before, immediately after as well as 1 ⁺ and 3-days ⁺ after	Post-race (hot temperature): Blood biochemistry: RBC (–); Hct (↓) ^a ; Hb (↑)*; MCV (↓) ^a ; MCH (↑) ^a ; MCHC (↑) ^a , EPO (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑)*; GGT (↓)*; Bilirubin (↑)*		Post-race (temperate temperature): Blood biochemistry: RBC (↓) ^a ; Hct (↓) ^a ; Hb (↓)*; MCV (–); MCH (↑) ^a ; MCHC (↑) ^a , EPO (↑)* Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; GGT (↑) ^a ; Bilirubin (↑) ^a	

				Immune response: WBC (↑) ^a ; Lymphocytes (↓) ^a , Monocytes (↑) ^a , Neutrophils (↑) ^a , Eosinophils (↑)*, Orosmuroid (↑) ^a , CRP (−) Iron panel: Ferritin (↑) ^a , Transferrin (↓)*, Iron (↑)* Cardiac muscle damage: cTnT (↑) ^a , proBNP (↑) ^a , Mb (↑) ^a Electrolytes: Na ⁺ (↑) ^a ; Mg ²⁺ (↓) ^a , K ⁺ (↑) ^a , Ca ²⁺ (−)			Immune response: WBC (↑) ^a ; Lymphocytes (↓) ^a , Monocytes (↑) ^a , Neutrophils (↑) ^a , Eosinophils (↓) ^a , Orosmuroid (↑) ^a , CRP (↓) ^a Iron panel: Ferritin (↑) ^a , Transferrin (↑) ^a , Iron (↑)* Cardiac muscle damage: cTnT (↑) ^a , proBNP (↑) ^a , Mb (↑) ^a Electrolytes: Na ⁺ (↑)*; Mg ²⁺ (↓) ^a , K ⁺ (↓)*, Ca ²⁺ (↑) ^a		
Sansoni, et al. [77]	Ultra-marathon (65 km)	17 male participants and 12 age matched, moderately active controls	Blood; before and immediately post-race	Post-race: Bone biochemistry: P1NP (↓) ^a ; Ga-OC (↑)*; Glu-OC (↓) ^a Hormonal response: Glucagon (↑) ^a ; GLP-1 (↑) ^a ; Resistin (↑) ^a ; Visfatin (↑) ^a ; Ghrelin (↑)*; GIP (↑)*Leptin (↓) ^a , C-peptide (↓) ^a ; Insulin (↓)*.					
Zakovska, et al. [78]	Ultra-marathon (100 km; freezing conditions)	15 participants (12 male; 3 female)	Blood; before and immediately post-race	Post-race: Blood cells: RBC (↓)*; Hct (↓)*; Hb (↓)*; PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; ALT (↓)* Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; IgA (↑)*; IgM (↑)*; IgG (↑) ^a ; Lymphocytes (↓)*; Eosinophils (↓)*					
Chlibkova, et al. [34]	Multistage ultra-marathon (n=7 consecutive marathon races)	26 participants (20 male; 6 female)	Blood and urine; before and after stages 1, 4 and 7	After stage 1: Blood biochemistry: Hct (↓) ^a Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (−), Urine: K ⁺ (↑) ^a ; Na ⁺ (↓) ^a		After stage 4: Blood biochemistry: Hct (↓) ^{a,c} Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (↑)* ^d , Urine: K ⁺ (↑) ^{a,d} ; Na ⁺ (↓)*		After stage 7: Blood biochemistry: Hct (↓) ^a Electrolytes: Plasma: K ⁺ (↑)*; Na ⁺ (↓)*, Urine: K ⁺ (↑) ^{a,c} ; Na ⁺ (−)	
Da Ponte, et al. [26]	Marathon (up-hill only)	22 male participants	Blood; before and immediately post-race	Post-race: Blood biochemistry: RBC (−); Hb (−); Hct (↓) ^a ; MCV (↓) ^a ; MCH (↓) ^a ; PL (↑) ^a ; TP (↑) ^a ; Alb (↑) ^a ; MCHC (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a Cardiac muscle damage: cTnI (↑) ^a ; Mb (↑) ^a Electrolytes: K ⁺ (↑) ^a ; Na ⁺ (↓)*; Cl [−] (↓) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; CRP (↑) ^a ; Lymphocytes (↓) ^a ; Eosinophils (↓) ^a ; Basophil (−)					
Li, et al. [79]	Ultra-marathon (400 km)	16 male participants	Blood; before and immediately after the race	Post-race: Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a					
Liu, et al. [27]	Ultra-marathon (±153.64 km; 24 h)	19 male participants	Blood; before and immediately after	Post-race: Blood biochemistry: Reticulocyte (↑) ^a ; RBC (↓) ^a ; Hct (↓) ^a ; Hb (↓) ^a ; Haptoglobin (↓) ^a ; MCV (↓)*; MCH (↓) ^a ; MCHC (−) Iron panel: Ferritin (↑) ^a ; Iron (↑)*					
Park, et al. [80]	Ultra-marathon (100 km)	22 male participants (11 with EIH and 11	Blood; before, immediately after,	Post-race: Cardiac muscle damage: cTnI (↑) ^a . NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a					

		with normal exercise blood pressure)	24 h ⁺ , 72 h ⁺ and 120 h ⁺ post-race	Immune response: CRP (↑) ^a		
Shin, et al. [81]	Ultra-marathon (622 km)	10 middle-age male participants	Blood; before, at 300 km, immediately after, as well as 3 ⁺ and 6-days ⁺ post-race	300 km of running: Blood biochemistry: RBC (↓) ^a , PL (↓) [*] , Hct (↓) [*] ; Hb (↓) ^a Cardiac muscle damage: cTnI (↑) ^a , NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: Hs-CRP (↑) ^a , WBC (↑) ^a	Post-race: Blood biochemistry: RBC (↓) ^a , PL (↑) [*] , Hct (↓) [*] ; Hb (↓) ^a Cardiac muscle damage: cTnI (↑) ^a , NT-proBNP (↑) ^a ; CK-MB (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Immune response: Hs-CRP (↑) ^a , WBC (↑) ^a	
Tirabassi, et al. [82]	Ultra-marathon (100 km)	36 participants (28 male; 8 female)	Blood; before and within 30 min after the race	Post-race: Blood biochemistry: TP (↓) ^a , Alb (↓) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a ; ALT (↑) ^a ; Bilirubin (↑) ^a Immune response: ALP (↓) [*]		
Aengevaeren, et al. [83]	Marathon	82 participants (65 male; 7 female)	Blood; before and immediately after the race	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; sST2 (↑) ^a		
Bekos, et al. [84]	Marathon	34 marathon participants (24 male; 10 female); 36 half-marathon participants (19 male; 17 female); 30 sedentary volunteers ⁺ (17 male; 13 female)	Blood; before, immediately after as well as 2–7 days ⁺ after the marathon	Half-marathon athletes: Immune response: HSP70 (↑) ^a ; HSP27 (↑) ^a , WBC (↑); PL (↑) – <i>In individuals showing signs of EIB</i>	Marathon athletes: Immune response: HSP70 (↑) ^a ; HSP27 (↑) ^a ; WBC (↑); PL (↑) – <i>In individuals showing signs of EIB</i>	
Górecka, et al. [85]	Ultra-marathon	10 male participants	Blood; before, immediately after as well as 90 min post-race ⁺	Post-race: Blood biochemistry: Angiopoietin-like protein 4 (↑) ^a		
Hoppel, et al. [86]	Ultra-marathon (67 km)	8 male participants	Blood; before, after as well as 24 h ⁺ post-race	Post-race: Blood biochemistry: RBC (↑) [*] , Hct (↑) [*] ; PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a Renal damage: BUN (↑) ^a Immune response: WBC (↑) ^a ; Neutrophils (↑) ^a ; Monocytes (↑) ^a ; CRP (↑) ^a ; Lymphocytes (↓) [*] ; Eosinophils (↓) [*] Electrolyte markers: Na ⁺ (↓) [*] ; K ⁺ (↑) [*] ; Cl ⁻ (↓) [*] ; Mg ²⁺ (↑) [*] ; Ca ²⁺ (↑) ^a ;		
Le Goff, et al. [87]	Marathon, half-marathon and 1 h duration exercise	23 male marathon participants, 15 male half-marathon participants and	Blood; before and immediately after each exercise as well as 3 h ⁺ post-race	1 h running: Blood cells: Hb (–) Muscle and hepatic damage: CK (↑)	Half-marathon: Blood cells: Hb (↑) [*] Muscle and hepatic damage: CK (↑)	Post-race: Blood cells: Hb (↑) [*] Muscle and hepatic damage: CK (↑)

		male 17 untrained runners ⁺		Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑) [*] ; NT-proBNP (↑) [*] ; Galactin-3 (↑) ^a ; CK-MB (↑) [*] ; Mb (↑) [*] Immune response: CRP (↑) [*] ; Myeloperoxidase (↑) [*] Renal damage: Cystatin (↑) [*]	Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑) [*] ; NT-proBNP (↑) ^a ; Galactin-3 (↑) ^a ; CK-MB (↑) [*] ; Mb (↑) [*] Immune response: CRP (↑) [*] ; Myeloperoxidase (↑) [*] Renal damage: Cystatin (↑) [*]	Cardiac muscle damage: cTnI (↑) ^a ; cTnT (↑) ^a ; BNP (↑) [*] ; NT-proBNP (↑) ^a ; Galactin-3 (↑) ^a ; CK-MB (↑) [*] ; Mb (↑) [*] Immune response: CRP (↑) [*] ; Myeloperoxidase (↑) [*] Renal damage: Cystatin (↑) [*]
Martin, et al. [88]	Marathon	21 participants (9 male; 13 female)	Blood; 24 h before, immediately after as well as 24 h ⁺ post-race ⁺	Post-race: Cardiac muscle damage: cTnI (↑) ^a ; BNP (↑) ^a		
Rubio-Arias, et al. [89]	Ultra-marathons (54 km and 11 km)	16 male athletes (54 km n=10; 111 km n=6)	Blood; before and immediately after, as well as 24 h ⁺ , 48 h ⁺ and 72 h ⁺ post-race	After 54 km ultra-marathon: Blood biochemistry: RBC (↑) [*] ; Hb (↑) [*] ; Hct (↑) [*] ; MCV (↑) [*] ; MCH (–); PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST (↑) ^a , ALT (↑) ^a , Mb (↑) ^a Cardiac muscle damage: cTnI (↑) ^a Immune response: WBC (↑) ^a , CRP (↓) [*]	After 111 km ultra-marathon: Blood biochemistry: RBC (↑) [*] ; Hb (↓) [*] ; Hct (↓) [*] ; MCV (↓) [*] ; MCH (↓) [*] , PL (↑) ^a Muscle and hepatic damage: CK (↑) ^a ; LDH (↑) ^a ; AST/GOT (↑) ^a , ALT/GPT (↑) ^a , Mb (↑) ^a Cardiac muscle damage: cTnI (↑) [*] Immune response: WBC (↑) ^a , CRP (↓) ^a	
Seal, et al. [43]	Ultra-marathon (246 km)	63 male participants	Blood: before, at 93 km and within 15 min after	93 km of running: Electrolytes: Na ⁺ (↓) ^a	Post-race: Electrolytes: Na ⁺ (↓) ^a	
Sierra, et al. [90]	Marathon	31 male participants	Sputum (induced); before, immediately after as well as 24 h ⁺ and 72 h ⁺ post-race	Airway immune response; post-race: Neutrophils (↑) ^a ; Macrophages (↑) ^a ; Epithelial cells (↑) ^a ; Lymphocytes (↓) [*] ; IL-6 (↓) [*] ; IL-8 (↓) [*] ; IL-23 (↓) ^a ; IL-33 (↓) ^a ; IL-12p40 (↓) ^a ; TSLP (↓) ^a		
Zebrowska, et al. [91]	Ultra-marathon (24 h; 149 km ± 33 km)	14 male participants	Blood; before, after marathon length as well as 12 h, and 48 h post-race ⁺	Marathon length: Cardiac muscle damage: NT-proBNP (↑) ^a ; CK-MB (↑) [*] ; cTnT (↑) [*] ; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑) [*]	12 h of running: Cardiovascular damage: NT-proBNP (↑) ^{*,c} ; CK-MB (↑) ^{*,b} ; cTnT (↑) ^{*,b} ; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑) ^{a,d}	Post-race: Cardiovascular damage: NT-proBNP (↑) ^{*,c} ; CK-MB (↑) ^{a,d} ; cTnT (↑) ^{a,d} ; H-FABP (↑); IMA (–) Immune response: Hs-CRP (↑) ^{a,d}
Malek, et al. [92]	Ultra-marathon (100 km)	18 participants (15 male; 3 female)	Blood; before and immediately post-race	Post-race: Cardiac muscle damage: cTnI (↑) ^a Immune response: Hs-CRP (↑) ^a		

Footnote: Biochemical changes are denoted as elevated (↑) or reduced (↓), with significance of these changes indicated as follows: significant (a) and non-significant (*) compared to baseline; non-significant/unreported elevations compared to preceding time point (b); non-significant/unreported reduction compared to preceding time point (c); significantly elevated compared to preceding time point (d), significantly reduced compared to preceding time point (e), unchanged concentration (–), data not included in table (+). **Abbreviations:** ACTH: adrenocorticotrophic

hormone; AGE: advance glycation end-products; Alb: albumin; ALP: alkaline phosphatase; ALT: alanine transferase; AST: aspartate transferase; BNP: brain natriuretic peptide; BUN: blood-urea-nitrogen; Ca^{2+} : calcium; CK: creatine kinase; CK-MB: creatine kinase-myocardial band; Cl^- : chloride; CO_2 : carbon dioxide; COMP: cartilage oligomeric matrix protein; CRP: C-reactive protein; cTnI: cardiac troponin I; cTnT: cardiac troponin T; CXT: cross-linked C-telopeptide of type I collagen; Dkk-1: dickkopf-1; EIH: exercise-induced hypertension; EPO: erythropoietin; esRAGE: endogenous secretory RAGE; Ga-OC: carboxylate osteocalcin; GGT: gamma-glutamyl transferase; GIP: gastric inhibitory polypeptide; GLP-1: glucagon-like peptide; Glu-OC: undercarboxylated osteocalcin; GM-CFS: granulocyte-macrophage colony-stimulating factor; Hb: hemoglobin; Hct: hematocrit; H-FABP: heart-type fatty acid-binding protein; HMGB1: high mobility group box 1; Hs-CRP: heat sensitive CRP; HSP: heat shock protein; ICTP: cross-linked carboxy-terminal telopeptide of type I collagen; $IFN-\gamma$: interferon-gamma; Ig: immunoglobulin; IL: interleukin; IMA: ischemia-modified albumin; K^+ : potassium; KIM-1: kidney injury molecule-1; LDH: lactate dehydrogenase; LPS: lipopolysaccharide; Mb: myoglobin; MCH: mean corpuscular hemoglobin volume; MCHC: mean corpus hemoglobin concentration; MCV: mean corpus volume; Mg^{2+} : magnesium; Na^+ : sodium; NGAL: neutrophil gelatinase-associated lipocalin; NT-proBNP: N-terminal pro-BNP; O_2 : oxygen; PINP: pro-collagen type I N-terminal pro-peptide; P^{3-} : phosphate; PICP: procollagen I carboxyterminal pro-peptide; PL: platelets; Pro-ANP: pro-atrial natriuretic peptide; RAGE: receptor of advance glycation end-products; RANKL: receptor activator of nuclear factor κB ligand; RBC: red blood cell; sST2: suppression of tumorigenicity 2; $TNF-\alpha$: tumor necrosis factor alpha; TP: total protein; TSLP: thymic stromal lymphopoietin; VEGF: vascular endothelial growth factor; WBC: white blood cells/leukocytes.

Table 2: Endurance running-induced metabolic biomarker adaptations reported in recent (2000–2020) literature, arranged according to the publication year.

Reference	Exercise type	Cohort	Sampling media	Metabolic adaptations	
Mastaloudis, et al. [153]	Ultra-marathon (50 km)	11 participants (8 male; 3 female)	Blood; fasting state, before, at 27 km, immediately after, 1 h ⁺ and 24 h ⁺ post-race	After 27 km of running: Lipid, ketones and cholesterol: Chol (–); TAG (↑)* ROS and vitamins: Urate (↑) ^a ; α-Tocopherol (↑) ^a ; Ascorbate (↑) ^a	Ultra-marathon length Lipid, ketones and cholesterol: Chol (–); TAG (↑)*,b; ROS and vitamins: Urate (↑) ^a ; α-Tocopherol (↑) ^a ; Ascorbate (↑) ^{a,b}
Kratz, et al. [49]	Marathon	37 participants (32 male; 5 female)	Blood; before, within 4 h after and 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Lipid, ketones and cholesterol: TAG (↓)*; Chol (↑)* ROS and vitamins: Urate (↑) ^a ; Cr (↑)	
Petibois, et al. [154]	10 km run	14 participants (<i>sex not provided</i>)	Blood; before and immediately post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a ; Lactate (↑) ^a Lipid, ketones and cholesterol: TAG (↓) ^a ; Glycerol (↑) ^a ; Urea (↑) ^a	
Uchakin, et al. [50]	Marathon (White Rock and Cowton marathons)	15 male participants	Blood; before, 20 min and 60 min after the race as well as 2 ⁺ , 5 ⁺ and 8-days ⁺ post-race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	
Niessner, et al. [40]	Marathon	19 participants (17 male; 2 female)	Blood; before and immediately after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	
Real, et al. [155]	Marathon	22 male participants	Blood; before and after the marathon	Post-race: Carbohydrates and TCA cycle: Glucose (–) Lipid, ketones and cholesterol: FA (↑) ^a ; TAG (↓) ^a Amino acids and urea cycle: Homocysteine (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ROS and vitamins: Folate (↑)*; Vit B12 (↓)*	
Leers, et al. [51]	Marathon	27 participants (25 male; 2 female)	Blood; before, immediately after and 24 h ⁺ post-race	Post-race: Amino acids and urea cycle: Urea (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a	
Kim, et al. [52]	Ultra-marathon (200 km)	54 male participants	Blood; before, after 100 km and immediately post-race	Post-race: Carbohydrates and TCA cycle: Lactate (↑)*; Glucose (↓)* Purine, pyrimidine and ATP: Cr (↑) ^a	
Mouzopoulos, et al. [53]	Ultra-marathon (245 km)	16 male participants	Blood; 5-days before, immediately after	Post-race: Amino acids and urea cycle: OH-proline (↓) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a	

			as well as 1 ⁺ , 3 ⁺ and 5-days ⁺ post-race			
Siegel, et al. [39]	Marathon	33 participants (<i>sex not provided</i>)	Blood; before and after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a		
Sport in Society Weibel, et al. [156]	Ultra-marathon (>65 km per day for 6 days)	31 participants	Urine; before and 4-20 times during the race	Post-race (average): Lipid, ketones and cholesterol: Ketones (↑) ^a (n=22); Ketones (↓) ^a (n=9)		
Lehmann, et al. [157]	Treadmill exercises (±14 km -16 km)	21 participants	Blood; before and immediately after exercise	Post-race: Carbohydrates and TCA cycle: Lactate (↑) ^a Lipid, ketones and cholesterol: Hippurate (↑) ^a ; C6:0 car. (↑) ^a ; C8:0 car. (↑) ^a ; C10:1 car. (↑) ^a ; C10:0 car. (↑) ^a ; C14:2 car. (↑) ^a ; C12:0 car. (↑) ^a		
Lewis, et al. [158]	Marathon and 60 min treadmill running exercises	25 marathon participants and 45 individuals (treadmill exercise)	Blood; before and after exercise	Treadmill exercise: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Malate (↑) ^a ; Succinate (↑) ^a ; Fumarate (↑) ^a ; Pyruvate (↑) ^a ; Glucose-6-phosphate (↑) ^a ; 3-Phosphoglycerate (↑) ^a Lipid, ketones and cholesterol: Glycerol (↑) ^a Amino acids and urea cycle: Alanine (↑) ^a ; Leucine (↑) ^a ; Serine (↓) ^a ; Glutamate (↓) ^a ; Cysteine (↓) ^a ; Homocysteine (↓) ^a ; Glutamine (↓) ^a ; Citrulline (↓) ^a Purine, pyrimidine and ATP: Inosine (↑) ^a ; Hypoxanthine (↑) ^a ; AMP (↑) ^a ; Xanthine (↑) ^a ; Allantoin (↓) ^a ; Niacin (↑) ^a ; Pantothenate (↑) ^a ; Cr (↑) ^a	Marathon distance: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Malate (↑) ^a ; Aconitate (↑) ^a ; α-ketoglutarate (↑) ^a ; Citrate (↑) ^a ; Succinate (↑) ^a ; Fumarate (↑) ^a ; Pyruvate (↑) ^a ; Glucose-6-phosphate (↑) ^a Lipid, ketones and cholesterol: Glycerol-3-phosphate (↑) ^a ; Glycerol (↑) ^a ; β-OH-butyrate (↑) ^a Amino acids and urea cycle: Leucine (↓) ^a ; Serine (↓) ^a ; Glutamine (↓) ^a ; Proline (↓) ^a ; Lysine (↓) ^a ; Threonine (↓) ^a ; Betaine (↓) ^a ; Asparagine (↓) ^a ; Dimethylglycine (↓) ^a ; Histidine (↓) ^a ; Kynurenate (↑) ^a ; Valine (↓) ^a ; Homovanillate (↑) ^a ; 4-OH-phenylpyruvate (↑) ^a ; Arginine (↓) ^a ; Quinolinate (↑) ^a ; Tryptophan (↓) ^a ; Citrulline (↓) ^a ; Ornithine (↓) ^a ; Argininosuccinate (↑) ^a Purine, pyrimidine and ATP: Hypoxanthine (↑) ^a ; AMP (↑) ^a ; IMP (↑) ^a ; Xanthine (↑) ^a ; Xanosine (↑) ^a ; Niacin (↑) ^a ; Pantothenate (↑) ^a ; Cr (↑) ^a	
McCullough, et al. [58]	Marathon	25 participants (12 male; 13 female)	Blood; before, after and 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a		
Turner, et al. [159]	Ultra-marathon (233 km)	9 male participants	Blood; before, immediately after as well as 24 h ⁺ , 7 ⁺ and 28-days ⁺ post-race	Post-race: ROS and vitamins: Glutathione (↑) ^a ; Lipid peroxides (↑)		
Waśkiewicz, et al. [60]	Ultra-marathon (168.5 km)	19 male participants	Blood; before, at marathon distance,	Post-race:	12 h of running:	Post-race:

			within 12 h and immediately after the ultra-marathon	Lipid, ketones and cholesterol: Chol (–); TAG (–); FA (↑) ^a ; Glycerol (↑) ^a ; β-OH-butyrate (↑)*	Lipid, ketones and cholesterol: Chol (–); TAG (–); FA (↑) ^{a,b} ; Glycerol (↑) ^{a,c} ; β-OH-butyrate (↑) ^{a,c}	Lipid, ketones and cholesterol: Chol (↓) ^a ; TAG (↓) ^a ; FA (↑) ^{a,c} ; Glycerol (↑) ^{a,c} ; β-OH-butyrate (↑) ^{a,b}
Klapcinska, et al. [61]	Ultra-marathon (48 h; 183–320 km)	7 male participants	Blood; before, after running 12 h, 24 h and within 10 min after the race as well as 24 h ⁺ and 48 h ⁺ post-race	12 h of running: Carbohydrates and TCA cycle: Lactate (↓)*; Glucose (↓)*	24 h of running: Carbohydrates and TCA cycle: Lactate (↓)* ^c ; Glucose (↑)*	Post-race: Carbohydrates and TCA cycle: Lactate (↓)* ^c ; Glucose (↑)* ^b
Nieman, et al. [160]	Repeated treadmill exercises (±25 km per day for 3 days)	15 participants (7 male; 8 female)	Blood; before and directly after the 3-day intensified training exercise	Post-exercise: Carbohydrates and TCA cycle: Succinate (↑) ^a ; Fumarate (↑) ^a ; Malate (↑) ^a Lipid, ketones and cholesterol: Palmitoleate (↑) ^a ; Myristoleate (↑) ^a ; 10-heptadecenoate (↑) ^a ; 10-nonadecenoate (↑) ^a ; Dihomolinoleate (↑) ^a ; Stearidonate (↑) ^a ; Myristate (↑) ^a ; Eicosenoate (↑) ^a ; Oleate (↑) ^a ; Margarate (↑) ^a ; Laurylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; 4-decenoylcar. (↑) ^a ; Dodecanedioate (↑) ^a ; Hexadecanedioate (↑) ^a ; Tetradecanedioate (↑) ^a ; Octadecanedioate (↑) ^a ; Linolenate (↑) ^a ; Docosapentaenoate (↑) ^a ; Linoleate (↑) ^a ; Eicosapentaenoate (↑) ^a ; Dihomolinolenate (↑) ^a ; Docosahexaenoate (↑) ^a Lysolipid, bile salts and steroids/hormones: 1-arachidonoylGPEA (↓) ^a ; 1-palmitoylGPEA (↓) ^a ; 1-oleoylGPEA (↓) ^a ; 2-linoleoylGPEA (↓) ^a ; Taurodeoxycholate (↓) ^a ; Taurocholate (↓) ^a ; Glycochenodeoxycholate (↓) ^a ; Glycodeoxycholate (↓) ^a ; Cholate (↓) ^a Glycocholate (↓) ^a ; 2-aminoadipate (↑) ^a Amino acids and urea cycle: 4-OH-phenyl-pyruvate (↑) ^a ; N-acetylphenylalanine (↑) ^a ; Isovalerylcar. (↑) ^a ; OH-isobutyrate (↑) ^a		
Daskalaki, et al. [161]	1 h treadmill/bicycle ergometer exercise	3 male participants	Urine; before (first pass) ⁺ , 1 h before ⁺ , immediately after as well as numerous time s within the preceding 24 h ⁺ post-exercise	Post-exercise: Carbohydrates and TCA cycle: Pyruvate (↑) ^a Purine, pyrimidine and ATP: N2-N2-Dimethylguanosine (↑)*; Xanthosine (↑) ^a ; Inosine (↑) ^a ; Deoxyinosine (↑) ^a ; Guanine (↑) ^a ; Hypoxanthine (↑) ^a Amino acids and urea cycle: 3-OH-tryptophan (↑)*; Xanthurenate isomer (↑) ^a ; Kynurenate (↑)*; OH-tryptophol (↓)*; N1-Methyl-2-pyridone-5-carboxamide (↑)* Tryptophan (↓)*; O-Acetyl-L-homoserine (↑) ^a ; Histidine (↓)*; Methionine (↑)*; Proline (↑)*; Acetylvanilalanine (↑) ^a ; N-(Carboxyethyl) arginine (↑) ^a ; N-Acetyl-D-tryptophan (↑) ^a ; N-acetylmethionine (↑)*; Indole pyruvate (↑)*; Acetamido-oxohexanoate (↑)*; OH-phenylpyruvate (↑)*; Oxoarginine (↑)*; Guanidovaleramide (↑) ^a ; AcetoOH-butanoate (↑) ^a ; Acetamidobutanoate (↑)*; DiOH-methylbutanoate (↑)*; Methyl-oxopentanoate (↑)*; Dioxopentanoate (↑)*; OH-pentanoate (↑)*; Methyloxobutanoate (↑)*; OH-butanoate(↑)* ROS and vitamins: Riboflavin (↑)*; Pantothenate (↑) ^a Lipid, ketones and cholesterol: Dodecenoylcar. (↑) ^a ; Undecanoylcar. (↑)*; Decanoylcar. (↑)*; L-Octanoylcar. (↑)*; Methylglutarylcar. (↑)*; Hexanoylcar. (↑)*; Valerylcar. (↑)*; DeOH-car. (↑) ^a		

				Lysolipid, bile salts and steroids/hormones: Urocortisol glc. (↑)*; Dihydrocortisone glc. (↑)*; Hydrocortisone sulphate (↑)*; OH-androsterone glc. (↑)*; Androstane diol glc. (↑)*; Androsterone glc. (↑)*; Oxoandrostane glc. (↑)*
Khodae, et al. [66]	Ultra-marathon (161 km)	20 participants pre-marathon (14 male; 6 female), 21 participants post-marathon (17 male; 4 female)	Blood; before and immediately after	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a
Mrakic-Sposta, et al. [162]	Ultra-marathon (330 km)	25 male participants	Blood and urine; before, during (±165 km) and immediately post-race	After 165 km of running: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Glucose (↑)* Lipid, ketones and cholesterol: Ketones (–) Purine, pyrimidine and ATP: Cr (–) ROS and vitamins: Neopterin (–); ROS production rate (↑) ^b ; 8-OH-2-deoxyguanosine (–); 8-isoprostane (–) Post-race: Carbohydrates and TCA cycle: Lactate (↑) ^a ; Glucose (↑)* Lipid, ketones and cholesterol: Ketones (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ROS and vitamins: Neopterin (↑) ^a ; ROS production rate (↑) ^a ; 8-OH-2-deoxyguanosine (↑) ^a ; 8-isoprostane (↑) ^a
Suzuki, et al. [69]	5 h running exercise (Participant A=34.14 km; participant B=43.05 km)	2 female participants	Blood; before, within 60 min after as well as 1 ⁺ , 3 and 5 h post-race	Participant A post-race: Carbohydrates and TCA cycle: Glucose (↑) Lysolipid, bile salts and steroids/hormones: Cortisol (↓); Adrenalin (↑); Noradrenalin (↑); Dopamine (↑) Lipid, ketones and cholesterol: TAG (↓), Acetoacetate (↑), 3-OH-butyrate (↑) Amino acids and urea cycle: Aspartate (↓); Glutamate (↑); Asparagine (↑); Serine (↑); Glutamine (↑); Glycine (↑); Histidine (↑); Arginine (↑); Taurine (↓); Threonine (↓); Alanine (↑); Proline (↑); Tyrosine (↑); Valine (↑); Methionine (↑); Isoleucine (↑); Leucine (↑); Phenylalanine (↑); Lysine (↑) Participant B post-race: Carbohydrates and TCA cycle: Glucose (↑) Lysolipid, bile salts and steroids/hormones: Cortisol (↑); Adrenalin (↑); Noradrenalin (↑); Dopamine (↑) Lipid, ketones and cholesterol: TAG (↓), Acetoacetate (↑), 3-OH-butyrate (↑) Amino acids and urea cycle: Aspartate (↓); Glutamate (↓); Asparagine (↓); Serine (↓); Glutamine (↓); Glycine (↓); Histidine (↓); Arginine (↓); Taurine (↓); Threonine (↓); Alanine (↓); Proline (↓); Tyrosine (↑); Valine (↓); Methionine (↑); Isoleucine (↓); Leucine (↑); Phenylalanine (↓); Lysine (↓)
Arakawa, et al. [70]	Ultra-marathon (130 km)	18 middle-aged participants	Blood; before, after 42 km (marathon), immediately after as well as 3 ⁺ , 5 ⁺ and 7-days ⁺ post-race	Marathon length: Lipid, ketones and cholesterol: FA (↑); TAG (↓)*; Chol (↑)*; HDL-C (↑)* Lysolipid, bile salts and steroids/hormones: Cortisol (↑)*; Adrenalin (↑) ^a ; Noradrenalin (↑) ^a ; Dopamine (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) Post-race: Lipid, ketones and cholesterol: FA (↑) ^a ; TAG (↓) ^a ; Chol (↓); HDL-C (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a ; Adrenalin (↑) ^{a,b} ; Noradrenalin (↑) ^{a,c} ; Dopamine (↑) ^c Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*
Bekos, et al. [71]	Marathon and half-marathon	34 marathon participants, 36 half-marathon	Blood and urine; before and immediately after	Half-marathon: Carbohydrates and TCA cycle: Lactate (↑) ^a Purine, pyrimidine and ATP: Urine Cr (–); Serum Cr (↑) ^a Marathon: Carbohydrates and TCA cycle: Lactate (↑) ^a Purine, pyrimidine and ATP: Urine Cr (–); Serum Cr (↑) ^a

		participants and 30 sedentary controls	as well as 2–7 days ⁺ post-race			
Shin, et al. [74]	Ultra-marathon (308 km)	15 participants (<i>sex not provided</i>)	Blood; before, at marathon distance, after 100 km and immediately post- race	Marathon length: Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) ^a	100 km of running: Purine, pyrimidine and ATP: Cr (↑) ^{a,d} ; Urate (↑) ^{a,d}	Post-race: Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) [*]
Traiperm, et al. [163]	Marathon	50 adolescent participants (30 male; 20 female)	Blood; before, immediately after as well as 24 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑) ^a Lipid, ketones and cholesterol: TAG (↓) ^a ; HDL (↓) ^a ; LDL (↓) ^a ; Chol (↓) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) [*]		
Vezzoli, et al. [164]	Ultra-marathons (50 km and 100 km)	24 participants (50 km n=6 [3 male; 3 female] and 100 km n=18 [2 male; 11 female])	Blood; before and immediately after the race	After 50 km: ROS and vitamins: Plasma: Cysteine (↑) [*] ; Homocysteine (↑) [*] ; Cysteinylglycine (↑) [*] ; Glutathione (↑) [*] ; TBARS (↑) ^a ; ROS (↑) ^a ; Erythrocyte: Cysteine (↑) [*] ; Homocysteine (↑) [*] ; Cysteinylglycine (↑) [*] ; Glutathione (↑) [*] ; 8-OH-2- deoxyguanosine (↑) ^a ; 8-isoprostane (↑) ^a		After 100 km: ROS and vitamins: Plasma: Cysteine (↑) ^a ; Homocysteine (↑) ^a ; Cysteinylglycine (↑) ^a ; Glutathione (↑) ^a ; TBARS (↑) ^a ; ROS (↑) ^a ; Erythrocyte: Cysteine (↑) [*] ; Homocysteine (↑) [*] ; Cysteinylglycine (↓) [*] ; Glutathione (↑) [*] ; 8-OH-2- deoxyguanosine (↑) ^a ; 8-isoprostane (↑) ^a
Yamada, et al. [165]	Ultra-marathon (135 km)	35 male participants	Blood; before, after 45 km and immediately post- race	45 km of running: Lipid, ketones and cholesterol: FA (↑) ^a Amino acids and urea cycle: Tryptophan (↓) [*] ; Serotonin (↑) ^a ; Kynurenine (↓) [*]		Post-race: Lipid, ketones and cholesterol: FA (↑) ^{a,d} Amino acids and urea cycle: Tryptophan (↓) ^a ; Serotonin (↓) ^{*c} ; Kynurenine (↑) ^{*d}
Nieman, et al. [166]	Treadmill exercise (±24.9 km)	24 male participants	Muscle biopsy and blood; before and after exercise	Post-exercise: Carbohydrates and TCA cycle: Glycogen (↓) ^a ; Succinate (↑) ^a ; Malate (↑) ^a Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a ; Adrenalin (↑) ^a Lipid, ketones and cholesterol: Glycerol (↑) ^a ; Myristate (↑) ^a ; Oleate (↑) ^a ; Margarate (↑) ^a ; 16-OH- palmitate (↑) ^a ; 10-heptadecenoate (↑) ^a ; Myristoleate (↑) ^a ; Palmitoleate (↑) ^a ; Palmitate (↑) ^a ; Dihomolinoleate (↑) ^a ; Eicosenoate (↑) ^a ; Laurate (↑) ^a ; 10-nonadecenoate (↑) ^a ; Linoleate (↑) ^a ; Linolenate (↑) ^a ; Hexadecanedioate (↑) ^a ; Stearate (↑) ^a ; Docosadienoate (↑) ^a ; Nonadecanoate (↑) ^a ; 5-dodecenoate (↑) ^a ; Stearidonate (↑) ^a ; Arachidate (↑) ^a ; Pentadecanoate (↑) ^a ; 3-OH-butyrate (↑) ^a ; Caprate (↑) ^a ; Decanoylcar. (↑) ^a ; Methionylalanine (↑) ^a ; Tetradecanedioate (↑) ^a ; Octanoylcar. (↑) ^a ; Acetoacetate (↑) ^a ; 3-OH-decanoate (↑) ^a ; Docosapentaenoate (↑) ^a ; 13/9-HODE (↑) ^a ; 17-methylstearate (↑) ^a ; Laurylcar. (↑) ^a ; Decenoylcarni-tine (↑) ^a ; Adrenate (↑) ^a ; Octadecanedioate (↑) ^a ; Erucate (↑) ^a ; 10-undecenoate (↑) ^a ; Methylpalmitate (↑) ^a ; 3-OH-octanoate (↑) ^a ; Dodecanedioate (↑) ^a		
Da Ponte, et al. [26]	Marathon (up-hill only)	22 male participants	Blood; before and immediately post- race	Post-race: Lysolipid, bile salts and steroids/hormones: Cortisol (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a		
Danese, et al. [75]	Half-marathon	30 participants (21 male; 9 female)	Blood; before and immediately after the race	Post-race: Lysolipid, bile salts and steroids/hormones: Cholate (↓) ^a ; Deoxycholate (↓) ^a ; Chenodeoxycholate (↓) ^a ; Ursodeoxycholate (↓) ^a ; Taurochenodeoxycholate (↑) [*] ; Glycocholate (↓) [*] ; Glycochenodeoxycholate (↓) [*] ; Glycoursodeoxycholate (–); Glycodeoxycholate (–); Hyodeoxycholate (↓) ^a ; Lithocholate (–); Taurocholate (–)		

Oliveira, et al. [4]	Marathon (hot and temperate temperatures)	26 male participants	Blood and urine, before, immediately after as well as 1 ⁺ and 3-days ⁺ after	Post-race (Heat): Amino acids and urea cycle: Urea (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑) ^a ; TBARS (↑) ^a ; SOD3 (↑) ^a ; Catalase (↓)*	Post-race (Temperate): Amino acids and urea cycle: Urea (↑) ^a Purine, pyrimidine and ATP: Cr (↑) ^a ; Urate (↑)*; TBARS (↑) ^a ; SOD3 (↓)*; Catalase (↓)*
Davison, et al. [167]	1 h treadmill exercise (75% VO _{2max} ; Hypoxic and normoxic conditions)	24 male participants	Blood; before, immediately after as well as 3 h post-exercise ⁺	Post-exercise (Hypoxia): Lipid, ketones and cholesterol: Propionylcar. (↑) ^a ; Butyrylcar. (↑) ^a ; 2-Methylbutyrylcar./Pivaloylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Undecenoylcar. (↑) ^a ; 4,8 dimethylnonanoyl car. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Tridecenolcar. (↑) ^a ; Tetradecadienoylcar. (↑) ^a ; cis-5-Tetradecenoylcar. (↑) ^a ; Hexadecatetraenoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadec-2-enoyl car. (↑) ^a ; Palmitoylcar. (↑) ^a ; Octadecatrienoylcar. (↑) ^a ; Elaidic car./Vaccenyl car. (↑) ^a ; Hippurate(↑) ^a ; Myristate (↑) ^a ; Palmitoleate (↑) ^a ; α-Linolenate (↑) ^a ; Linoleate(↑) ^a ; Arachidonate (↑) ^a	Post-exercise (Normoxia): Lipid, ketones and cholesterol: Propionylcar. (↑) ^a ; Butyrylcar. (↑) ^a ; 2-Methylbutyrylcar./Pivaloylcar. (↑) ^a ; Hexanoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Undecenoylcar. (↑) ^a ; 4,8 dimethylnonanoyl car. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Tridecenolcar. (↑) ^a ; Tetradecadienoylcar. (↑) ^a ; cis-5-Tetradecenoylcar. (↑) ^a ; Hexadecatetraenoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadec-2-enoyl car. (↑) ^a ; Palmitoylcar. (↑) ^a ; Octadecatrienoylcar. (↑) ^a ; Elaidic car./Vaccenyl car. (↑) ^a ; Hippurate (↑) ^a ; Myristate (↑) ^a ; Palmitoleate (↑) ^a ; α-Linolenate (↑) ^a ; Linoleate (↑) ^a ; Arachidonate (↑) ^a
Howe, et al. [168]	80.5 km treadmill exercise (self-paced)	9 male participants	Blood; before and immediately post-exercise	Post-exercise: Carbohydrates and TCA cycle: Rhamnose (↓) ^a ; Galactosamine (↓) ^a ; Hexose (↓) ^a Lipid, ketones and cholesterol: Acetylcar. (↑) ^a ; Propanoylcar. (↑) ^a ; Butanoylcar. (↑) ^a ; Hexenoylcar. (↑) ^a ; Hexanoylcar.isomer (↑) ^a ; Heptanoylcar. (↑) ^a ; Octenoylcar. (↑) ^a ; Octanoylcar. (↑) ^a ; Nonanoylcar. (↑) ^a ; Decadienoylcar. (↑) ^a ; Decenoylcar. (↑) ^a ; Decanoylcar. (↑) ^a ; Keto-decanoylcar. (↑) ^a ; Dimethylnonanoylcar. (↑) ^a ; Dodecenoylcar. (↑) ^a ; Hydroxydodecanoylcar. (↑) ^a ; Tetradecadienylcar.isomer (↑) ^a ; Tetradecanoylcar. (↑) ^a ; Hydroxytetradecadienylcar. (↑) ^a ; Hydroxytetradecenoylcar. (↑) ^a ; Hydroxymyristoylcar. (↑) ^a ; Hexadecadienoylcar. (↑) ^a ; Hexadecenoylcar. (↑) ^a ; Palmitoylcar. (↑) ^a ; Hydroxyhexadecadienoylcar. (↑) ^a ; Hydroxyhexadecenoylcar. (↑) ^a ; Octadecadienoylcar. (↑) ^a ; Hexadecanedioicacid monocar. ester (↑) ^a ; Decanoate (↑) ^a ; Dodecadienoate (↑) ^a ; Dodecanoate (↑) ^a ; Decanedioate (↑) ^a ; Hydroxydodecatrienoate; Tridecenoate (↑) ^a ; Tetradecadienoate (↑) ^a ; Tetradecenoate iso (↑) ^a ; Tetradecenoate iso (↑) ^a ; Dodecanedioate (↑) ^a ; Hydroxytetradecadienoate (↑) ^a ; Pentadecenoate (↑) ^a ; Hydroxytetradecadienoate (↑) ^a ; Hydroxytetradecanoate iso (↑) ^a ; Hydroxytetradecanoate iso (↑) ^a ; Hexadecadienoate iso (↑) ^a ; Hexadecadienoate iso (↑) ^a ; Palmitoleate (↑) ^a ; Tetradecanedioate (↑) ^a ; Hydroxyhexadecatrienoate (↑) ^a ; Hydroxyhexadecadienoate (↑) ^a ; Heptadecenoate (↑) ^a ; Hydroxyhexadecenoate iso (↑) ^a ; Hydroxyhexadecenoate (↑) ^a ; Hydroxyhexadecanoate (↑) ^a ; Octadecatetraenoate (↑) ^a ; Linolenate (↑) ^a ; Linoleate (↑) ^a ; Oleate (↑) ^a ; Dihydroxyhexadecadienoate (↑) ^a ; Dihydroxyhexdecenoate (↑) ^a ; Hydroxyoctadecadienoate (↑) ^a ; Hydroxyoctdecenoate (↑) ^a ; Dihydroxyoctadecanoate (↑) ^a ; Nitrooctadecenoate (↑) ^a ; Docosahexaenoate (↑) ^a ; Docosapentaenoate (↑) ^a ; Docosatetraenoate (↑) ^a Amino acids and urea cycle: Glycine (↓) ^a ; Alanine (↓) ^a ; 3-Amino-isobutanoate (↓) ^a ; Serine (↓) ^a ; Pyrrole-2-carboxylate (↓) ^a ; Proline (↓) ^a ; Oxopentanoic acid (↓) ^a ; Guanidinoacetate (↓) ^a ; Valine (↓) ^a ;	

				Betaine (↓)^a; Hydroxypentanoate (↑)^a; Threonine (↓)^a; Taurine (↓)^a; 5-Oxoproline (↓)^a; Hydroxyproline (↓)^a; Leucine (↓)^a; Isoleucine (↓)^a; Hydroxyhexanoic acid (↑)^a; Asparagine (↓)^a; Ornithine (↓)^a; Urocanate (↓)^a; Glutamine (↓)^a; Lysine (↓)^a; Glutamate (↓)^a; Methionine (↓)^a; Imidazol-5-yl-pyruvate (↓)^a; Indole-3-acetaldehyde (↓)^a; O-Acetylhomoserine (↓)^a; Arginine (↓)^a; Citrulline (↓)^a; Tyrosine (↓)^a; Hydroxyphenyllactate (↓)^a; Acetyl-L-lysine (↓)^a; Kynurenate (↑)^a; Tryptophan (↓)^a; Formylhydroxykynurenamine (↓)^a; Hydroxyindolepyruvate (↑)^a; 1-Methylnicotinamide (↓)^a Lysolipid, bile salts and steroids/hormones: Hydrocortisone (↑)^a; Urocortisone (↑)^a; Hydroxycholanate (↓)^a ; Deoxycholanoate (↓)^a; Chenodeoxyglycocholate (↓)^a; Glycocholate (↓)^a; Taurocholate (↓)^a; Chenodeoxycholate glc. (↓)^a; Cholestane—tetrol-glc. (↓)^a ROS and vitamins: γ-Tocopherol (↓)^a; α-Tocopherol (↓)^a Purine, pyrimidine and ATP: Hypoxanthine (↑)^a; Uridine (↓)^a; Pseudouridine (↓)^a
Li, et al. [79]	Ultra-marathon (400 km)	16 male participants	Blood; before and immediately after the race	Post-race: Amino acids and urea cycle: Alanine (↓)^a; Proline (↓)^a; Valine (↓)^a; Leucine (↓)^a; Isoleucine (↓)^a; Ornithine (↓)^a; Citrulline (↓)^a; Arginine (↑)[*]; Tryptophan (↓)[*]; Histidine (↑)[*]; Lysine (↓)^a; Methionine (↑)^a; Phenylalanine (↑)^a; Threonine (↓)^a; Glycine (↓)^a; Serine (↓)[*]; Taurine (↑)^a; Aspartate (↑)[*]; Glutamine (↑)[*]; Glutamate (↑)[*]; Cystathionine (↑)^a; Cystine (↑)^a; Homocysteine (−); 1-Methyl-L-histidine(↓)[*]; 3-Methyl-L-histidine (↑)[*]; α-Aminonbutyrate (↓)[*]; Argininosuccinate (↑)^a; β-Alanine (↑)[*]; Carnosine (↑)^a; Ethanolamine (↑)[*]; Homocitrulline (↓)[*]; OH-lysine (↓)[*]; Phosphorylethanolamine (↑)^a; Tyrosine (↑)^a; α-Aminoadipate (↑)[*]; Asparaginate (↓)[*]; Phosphoserine (↓)^a; Sarcosin (↓)^a Lysolipid, bile salts and steroids/hormones: Testosterone (↓)^a; Progesterone (↓)^a; 17-α-OH-progesterone (↓)^a; 11-Deoxycortisol (↓)[*]; 11-Deoxycorticosterone (↓)[*]; Cortisol (↑)^a; Aldosterone (↑)^a; Dehydroepiandrosterone (↓)[*]; Dehydroepiandrosterone sulfate (↑)^a; 4-Androstenedione (↓)^a; Dihydrotestosterone (↓)[*]; Corticosterone (↑)[*]; Cortisone (↓)[*]; Estrone (↑)^a; Estradiol (↑)[*]
Stander, et al. [169]	Marathon	31 participants (19 male; 12 female)	Blood; before and immediately post-race	Post-race: Carbohydrates and TCA cycle: Arabitol (↑)^a; Citrate (↑)^a; Erythritol (↑)^a; Fumarate (↑)^a; Glucarate (↑)^a; Glucose (↑)^a; Malate (↑)^a; Mannitol (↑)^a; Mannose (↑)^a; Myo-inositol (↑)^a; Pyruvate (↑)^a; Sorbose (↑)^a; Succinate (↑)^a; Tagatose (↑)^a; Threonate (↑)^a Lipid, ketones and cholesterol: α-OH-octanoate (↑)^a; α-Linolenate (↑)^a; β-OH-butyrate (↑)^a; OH-hexanoate (↑)^a; β-OH-α,β-didehydrosebacate (↑)^a ; 5-Dodecenoate (↑)^a; 5-Pregnene-3β,20α-diol (↑)^a; 10-Heptadecenoate (↑)^a; 11-Eicosenoate (↑)^a; 11,14-Eicosadienoate (↑)^a; Acetoacetate (↑)^a; Glycerol (↑)^a; Heptadecanoate (↑)^a; Laurate (↑)^a; Linoleate (↑)^a; Malonate (↑)^a; Myristoleate (↑)^a; Oleate (↑)^a; Palmitate (↑)^a; Palmitoleate (↑)^a; Pentadecanoate (↑)^a; Monopalmitin (↑)^a; Squalene (↑)^a; Tridecanoate (↑)^a Amino acids and urea cycle: α-Aminomalonate (↑)^a; α-Ethylhydracrylate (↑)^a; β-OH-isobutyrate (↑)^a; β-OH-isovalerate (↑)^a; β-OH-propionate (↑)^a; Alanine (↓)^a; Aspartate (↓)^a; Dimethylglycine (↓)^a; Glutarate (↓)^a; Glycerate (↑)^a; Glycine (↓)^a; Indole-3-acetate (↓)^a; Leucine (↓)^a; Methionine (↓)^a; Phenylalanine (↓)^a; p-OH-phenylacetate (↑)^a; p-OH-phenyllactate (↑)^a; Pyroglutamate (↓)^a; Serine (↓)^a; Threonine (↓)^a; Tyrosine (↓)^a; Valine (↓)^a
Górecka, et al. [85]	Ultra-marathon	10 male participants	Blood; before, immediately after	Post-race: Lipid, ketones and cholesterol: Glycerol (↑)^a; TAG (↓)^a; Chol (↑)[*]; FA (↑)^a

			as well as 90 min post-race ⁺	
Malek, et al. [92]	Ultra-marathon (100 km)	18 participants (15 male; 3 female)	Blood; before and immediately post-race	Post-race: Carbohydrates and TCA cycle: Glucose (↑)*; Lactate (↑)*
Schader, et al. [170]	Marathon	76 male participants	Blood; before (one week), immediately after as well as 24 h ⁺ and 72 h ⁺ post-race	Post-race: Carbohydrates and TCA cycle: Hexoses average (↓) ^a Lipid, ketones and cholesterol: C2:0–C18:0 car. (↑) ^a ; C2:1–C6:1 car. (↑) ^a C10:1–C18:1 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C10:2 car. (↑) ^a ; C14:2 car. (↑) ^a ; C14:2OH car. (↑) ^a ; C16:2 car. (↑) ^a ; C16OH car. (↑) ^a ; C18:2 car. (↑) ^a ; C18:1OH car. (↑) ^a ; C3OH car. (↑) ^a ; C5OH car. (↑) ^a ; C6 car. (↑) ^a ; LysoPC C14:0 (↓) ^a ; LysoPC C16:0 (↓) ^a ; LysoPC C16:1 (↓) ^a ; LysoPC C17:0 (↓) ^a ; LysoPC C18:0 (↓) ^a ; LysoPC C18:1 (↓) ^a ; LysoPC C18:2 (↓) ^a ; LysoPC C20:3 (↓) ^a ; LysoPC C20:4 (↓) ^a ; LysoPC C24:0 (↓) ^a ; LysoPC C28:0 (↓) ^a ; LysoPC C28:1 (↓) ^a ; PC C26–C44 (↓) ^a ; SMOH C14:1 (↓) ^a ; SMOH C16: (↓) ^a ; SMOH C22:1 (↓) ^a ; SMOH C22:2 (↓) ^a ; SMOH C24:1 (↓) ^a ; SMOH C16:0 (↓) ^a ; SMOH C18:0 (↓) ^a ; SMOH C24:0 (↓) ^a ; SM C16:1 (↓) ^a SM C18:1 (↓) ^a SM C20:2 (↓) ^a SM C22:3 (↓) ^a SM C24:1 (↓) ^a SM C26:1 (↓) ^a Amino acids and urea cycle: Alanine (↓) ^a ; Arginine (↓) ^a ; Glutamine (↓) ^a ; Glutamate (↑) ^a ; Histidine (↓) ^a ; Leucine (↓) ^a ; Lysine(↓) ^a ; Methionine (↓) ^a ; Ornithine (↑) ^a ; Citrulline (↓) ^a ; Phenylalanine (↓) ^a ; Proline (↓) ^a ; Serine (↓) ^a ; Threonate (↓) ^a ; Tryptophan (↓) ^a ; Tyrosine (↓) ^a ; Valine (↓) ^a

Footnote: Significance of adaptations are indicated as follows: significant (^a) and non-significant (*) compared to baseline; non-significantly/unreported elevations compared to preceding time point (^b); non-significantly/unreported reduction compared to preceding time point (^c); significantly elevated compared to preceding time point (^d), significantly reduced compared to preceding time point (^d), unchanged concentration (–), data not included in the table (⁺). **Abbreviations:** AMP: adenosine monophosphate; ATP: adenosine triphosphate; Car: carnitine; Chol: cholesterol; Cr: creatine; FA: fatty acids; Glc: glucuronide; GPEA: glycerophosphoethanolamine; HDL: high-density lipoprotein; Iso: isomer; LDL: low-density lipoprotein; OH: hydroxy; PC: Phosphatidylcholine; ROS: reactive oxygen species; SOD: superoxide dismutase; SM: Sphingomyelins; TAG: triacylglycerol; TBARS: thiobarbituric acid reactive substances; TCA: Tricarboxylic acid; Vit: vitamin

Table 3: *Proposed proficiency classification system for endurance running investigations.*

Investigative distance level	Novice athletes (0≤2yrs experience)		Intermediate athletes (3<5yrs experience)		Master athletes (5<10yrs experience)		Elite athletes (≥10yrs experience)	
	Number of competitive endurance races	Weekly training (km)	Number of competitive endurance races	Weekly training (km)	Number of competitive endurance races	Weekly training (km)	Number of competitive endurance races	Weekly training (km)
Level 1 (0<15 km)	0–2	0.0–3.5	3–5	3.5<7.0	5–10	7.0<10.5	10≤	≥10.5
Level 2 (15<30 km)		<18.5		18.5<22.0		22.0<25.5		≥25.5
Level 3 (30<50 km)		<35.0		35.0<40.0		40.0<45.0		≥45.0
Level 4 (50<80 km)		<57.5		57.5<65.0		65.0<72.5		≥72.5
Level 5 (80<150 km)		<87.5		87.5<95.0		95.0<102.5		≥102.5
Level 6 (150<300 km)		<180.7		180.7<225.0		225.0<262.5		≥262.5
Level 7 (300 km≤)		<350.0		350.0<400.0		400.0<450.0		≥450.0



The altered human serum metabolome induced by a marathon

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Abstract

Introduction Endurance races have been associated with a substantial amount of adverse effects which could lead to chronic disease and long-term performance impairment. However, little is known about the holistic metabolic changes occurring within the serum metabolome of athletes after the completion of a marathon.

Objectives Considering this, the aim of this study was to better characterize the acute metabolic changes induced by a marathon.

Methods Using an untargeted two dimensional gas chromatography time-of-flight mass spectrometry metabolomics approach, pre- and post-marathon serum samples of 31 athletes were analyzed and compared to identify those metabolites varying the most after the marathon perturbation.

Results Principle component analysis of the comparative groups indicated natural differentiation due to variation in the total metabolite profiles. Elevated concentrations of carbohydrates, fatty acids, tricarboxylic acid cycle intermediates, ketones and reduced concentrations of amino acids indicated a metabolic shift between various fuel substrate systems. Additionally, elevated odd-chain fatty acids and α -hydroxy acids indicated the utilization of α -oxidation and autophagy as alternative energy-producing mechanisms. Adaptations in gut microbe-associated markers were also observed and correlated with the metabolic flexibility of the athlete.

Conclusion From these results it is evident that a marathon places immense strain on the energy-producing pathways of the athlete, leading to extensive protein degradation, oxidative stress, mammalian target of rapamycin complex 1 inhibition and autophagy. A better understanding of this metabolic shift could provide new insights for optimizing athletic performance, developing more efficient nutrition regimens and identify strategies to improve recovery.

Keywords Marathon · Serum · Metabolomics · Metabolite markers · Fuel substrates

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1 Introduction

Although physical activity has been proven to be substantially beneficial to human health (Ojiambo 2013), various negative effects including cardiovascular dysfunction (Webner et al. 2012), muscle damage (Howatson et al. 2010), increased propensity to upper respiratory tract infections (Robson-Ansley et al. 2012) and severe inflammation (Bonasia et al. 2015) have been associated with running extensive distances, such as that of endurance races. Even though these races have become increasingly popular, limited research is based on the elucidation of the effects of these races using a metabolomics approach. Metabolomics is defined as the identification and quantification of the small metabolites (< 1500 Da) present in a biological system in order to determine the physiological effects induced by a specific perturbation (Heaney et al. 2017). Since metabolites are typically the end-products of the genome, transcriptome and proteome, alterations in these are indicative of the overall physiological state of the investigated biological system (Heaney et al. 2017).

Previous metabolomics studies have indicated elevated concentrations of various carbohydrate/glycolysis intermediates, which suggest the utilization of free glucose as the preferred energy source during strenuous physical activity (Lewis et al. 2010; Salway 2012; Waśkiewicz et al. 2012). Furthermore, significant alterations to the tricarboxylic acid (TCA) cycle intermediates were induced by a marathon (Turer et al. 2014) and could be attributed to additional strain placed on the electron transport chain (ETC), causing an imbalanced NADH:NAD⁺ ratio (Esterhuizen et al. 2017). According to previous work (Stellingwerff 2012), free glucose and other carbohydrate stores can become depleted within approximately 90 min after the start of the race, which most likely lead to the utilization of alternative fuel substrates (lipids and amino acids) for energy production (Waśkiewicz et al. 2012). Increased lipolysis activity results in elevated serum glycerol and free fatty acids (Lewis et al. 2010; Waśkiewicz et al. 2012), the latter of which produce acetyl-CoA via β -oxidation and subsequent energy via the TCA cycle and ETC (Salway 2012). Furthermore, the increased synthesis of acetyl-CoA could also ascribe for the elevated ketone concentrations previously reported (Pechlivanis et al. 2010), as it is a key component of ketogenesis. In the event that the strenuous physical activity continues beyond the capacity of the athlete's lipid stores, or if the traditional lipid oxidation pathways become saturated (Staron et al. 1989), the athlete's metabolism shifts towards protein catabolism (resulting in reduced blood amino acid levels) in an attempt to synthesize the energy required to complete the marathon (Lewis et al. 2010). These amino acids are primarily oxidized to

pyruvic acid and acetyl-CoA, both of which can serve as TCA cycle influx substrates for energy production (Salway 2012). Additionally, protein degradation has been shown to alter purine catabolism, resulting in elevated adenosine-monophosphate, inosine-monophosphate, hypoxanthine, xanthine, uric acid and allantoin (Turer et al. 2014), the latter of which is a uric acid derivative and a surrogate index of oxidative stress (Lewis et al. 2010).

Although these metabolomics studies provide some clues to the metabolic alterations that occur during strenuous physical activity such as endurance races, very few of these employed an untargeted metabolomics approach. Considering this, an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) metabolomics approach was used to holistically compare the serum metabolite profiles of 31 recreational marathon athletes before and after the completion of a marathon (42 km), in order to better characterize the acute metabolic changes induced by exercise stress.

2 Materials and methods

2.1 Participants

All participants completed a health and dietary questionnaire (including a menstrual cycle questionnaire for female participants) prior to the marathon in order to assess their eligibility. Individuals with food allergies, cardiovascular complications, musculoskeletal disorders/injuries, or those receiving anti-inflammatory treatment were excluded from the study. Athlete participation in this investigation was completely voluntary, and all the participants gave written and informed consent. A summary of the participant characteristics is presented in Table 1.

2.2 Clinical samples

Blood samples were collected by antecubital fossa venesection of 31 marathon athletes (19 males and 12 females) 24 h before and immediately after completing the Druridge Bay Marathon (Northumberland, UK). Pre-marathon samples

Table 1 Summary of the participant demographical information

Participant demographical information	Average $\pm$ standard deviation
Age (years)	41 $\pm$ 12
Pre-marathon athlete weight (kg)	71.3 $\pm$ 10.1
Post-marathon athlete weight (kg)	69.2 $\pm$ 9.7
Marathon experience (years)	9 $\pm$ 8
Marathon experience (races)	16 $\pm$ 29
Finishing time (hh:mm:ss)	04:19:09 $\pm$ 00:49:01

were collected the day preceding the race (between 10 am and 6 pm) as a means of reducing additional metabolic changes induced by the venesection stress as well as to limit interference to the athletes' pre-marathon regimens. The individuals were required to be in a hydrated, yet fasted state (for a minimum of 2 h) at time of baseline sample venesection. The samples were collected in standard 10 mL vacuum vials, placed on ice and transported to the laboratory (Faculty of Health and Life Sciences, Department of Sport, Exercise and Rehabilitation at the Northumbria University in Newcastle upon Tyne, UK) for immediate processing. Briefly, the blood was allowed to clot for 30 min and centrifuged at $3000\times g$ for 10 min. The supernatant (serum) was then extracted and immediately frozen ($-80\text{ }^{\circ}\text{C}$) before being transported (on dry ice) to the North-West University, Human Metabolomics: Laboratory of Infectious and Acquired Diseases. These serum samples were stored at $-80\text{ }^{\circ}\text{C}$ until metabolomics analyses commenced.

2.3 Chemicals and reagents

Methoxamine hydrochloride, 3-phenylbutyric acid and bis(trimethylsilyl)-trifluoroacetamide (BSTFA) with 1% trimethylchlorosilane (TMCS) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA) and the pyridine was from Merck (Darmstadt, Germany). The acetonitrile used, was an ultra-pure Burdick and Jackson brand (Honeywell International Inc., Muskegon, Michigan, USA) and hence did not require any further purification.

2.4 Sample extraction and derivatization

A total metabolome extraction was performed on all the collected serum samples, along with appropriate quality control (QC) samples. During this process, 50 μL of internal standard (3-phenylbutyric acid dissolved in a chloroform:methanol:water [1:3:1] solution; 0.45 $\mu\text{g}/\text{mL}$) was added to 50 μL of each serum sample. Hereafter, the samples were placed on ice before 300 μL of acetonitrile was added as a protein precipitation agent. Samples were then subjected to the REAX D-91126 vortex (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany) for 2 min and centrifuged at $3500\times g$ for 10 min at $4\text{ }^{\circ}\text{C}$. The supernatant of the biphasic solution was then transferred to a clean GC-MS sample vial and dried at $40\text{ }^{\circ}\text{C}$ under a light stream of nitrogen gas for approximately 45 min. Hereafter, 25 μL of methoxamine hydrochloride dissolved in pyridine (15 mg/mL) was added to each sample and incubated at $50\text{ }^{\circ}\text{C}$ for 90 min. Thereafter, 40 μL BSTFA with 1% TMCS was added, followed by derivatization for 60 min at $60\text{ }^{\circ}\text{C}$. Each derivatized sample was transferred to a new GC-MS vial containing a vial insert, and capped.

2.5 GCxGC-TOFMS analysis and processing

The derivatized samples were placed on a multi-purpose auto-sampler tray (Gerstel GmbH and co. KG, Mülheim van der Ruhr, Germany) in a randomized order, and analyzed using a Pegasus 4D GCxGC-TOFMS system (LECO Africa (Pty) Ltd, Johannesburg, South Africa), fitted with an Agilent 7890A GC and TOFMS (LECO Africa). During analyses, 1 μL of each sample was injected using a 1:3 split ratio. Purified helium was used as a carrier gas and set at a constant flow rate of 1 mL/min , while the injector temperature was set to operate at a constant $270\text{ }^{\circ}\text{C}$ throughout the entire sample analysis. The primary oven was fitted with a Restek Rxi-5MS capillary column (30 m; 0.25 μm diameter and 0.25 μm film thickness) and programmed to start the run at an initial temperature of $70\text{ }^{\circ}\text{C}$ for 2 min, followed by an increase of $4\text{ }^{\circ}\text{C}/\text{min}$ until a final temperature of $300\text{ }^{\circ}\text{C}$ was reached and maintained for 2 min. The secondary oven, equipped with a Restek Rxi-17 capillary column (1 m; 0.25 μm diameter and 0.25 μm film thickness), was set at an initial temperature of $85\text{ }^{\circ}\text{C}$, which was increased by $4.5\text{ }^{\circ}\text{C}/\text{min}$ until a final temperature of $300\text{ }^{\circ}\text{C}$ was reached and maintained for 2 min. The thermal modulator was set to pulse streams of cold and hot nitrogen gas every 3 s, for 0.5 s. The detector was set to disregard all mass spectra information for the first 400 s of each run to exclude solvent detection; however, this was still included on the time axis of the primary column to reflect accurate retention times. Additionally, the transfer line and ion source were respectively held at a constant of $270\text{ }^{\circ}\text{C}$ and $220\text{ }^{\circ}\text{C}$ for the entire run, with a detector voltage of 1600 V and filament bias of -70 eV . Mass spectra were acquired at a rate of 200 ms/s , over a range of 50–800 m/z . The total run time per sample was 111.28 min. Following GCxGC-TOFMS analysis, 838 peaks were identified, which was processed using LECO Corporation's ChromaTOF software (version 4.32), as described by Luies and Loots (2016).

2.6 Statistical analyses

Prior to statistical analysis, the dataset was normalized in relation to the internal standard and subjected to several "clean-up steps", including a 50% zero filter, QC drift correction, QC coefficient of variation filter and zero value replacement (Fernandez et al. 2000; Luies and Loots 2016). Hereafter, a natural shifted log transformation was performed to correct for skewed variable distribution, as well as auto scaling to align all variables (Van den Berg et al. 2006) (exclusively during multivariate analysis).

Both multivariate and univariate statistical analyses were performed using MATLAB software (2012), adjusted with the PLS toolbox (2016), to identify the metabolite markers best describing the variation between the comparative

groups. Multivariate approaches included principle component analysis (PCA) to determine whether a natural differentiation occurred between the comparative groups, and partial least square-discriminant analysis (PLS-DA) to characterize the group separation (Luies and Loots 2016). The univariate data analyses included a Wilcoxon signed rank test, corrected for multiple testing by the Benjamini–Hochberg procedure, to assess the statistical significance of each compound (Benjamini and Hochberg 1995; Rosner et al. 2006), while an independent effect size calculation (Wilcoxon signed effect size) was used to assess the practical significance of each detected metabolite (Tomczak and Tomczak 2014).

3 Results

The PCA differentiation between serum metabolite profiles (Fig. 1) of the marathon athletes before and after the completion of the marathon was clearly defined. The total amount of variance explained by the first three principle components (PCs) (R^2X cum) was 53%, of which PC1 accounted for 24%, PC2 for 20% and PC3 for 9%. Additionally, the PLS-DA model (results not shown) showed a modelling parameter R^2Y (cum) of 48.81% for the total variance of the response Y , and a Q^2 (cum) of 83.37%, indicating the cross-validation variation due to the response Y .

Since the aim of this study was to attain a holistic view of the altered human serum metabolome induced by a marathon, compounds with a PCA power value ≥ 0.5 or

Wilcoxon p -value ≤ 0.017 (BH-critical value) or an effect size ω -value ≥ 0.3 were considered significant and interpreted based on their associated metabolic/biochemical pathways. This multi-statistical approach yielded an initial list of 78 metabolite markers, of which 70 metabolite markers could be annotated by comparison of their mass spectra and retention times to that of commercially available and in-house libraries developed from previously injected standards (see Table 2).

4 Discussion

The altered metabolite markers listed in Table 2 are indicative of the major metabolic pathways affected by the marathon and are mainly associated with the macro-fuel substrate utilization pathways (carbohydrates, lipids and amino acids) and the regulation thereof (TCA, oxidative phosphorylation [OXPHOS] and gut microbiome). These metabolite pathways, along with the intermittent dietary-associated metabolite markers, are comprehensively discussed below and schematically presented in Fig. 2.

Various carbohydrate metabolites were significantly elevated following the marathon and can be ascribed to gluconeogenic influx (MacLaren and Morton 2012) and a reduced insulin secretion (Richter et al. 1992), which is typically induced by an initial depletion of glucose and glycogen stores occurring approximately 90 min after the start of a marathon (exercise intensity dependent) at a $VO_{2max} > 75\%$ (Stellingwerff 2012). The reduced insulin concentrations

Fig. 1 Principle component analysis scores plot showing clear differentiation of the serum samples of marathon athletes before (denoted by circles) and after (denoted by squares) the completion of a marathon. The variance accounted for are indicated in parenthesis. *PC* principle component

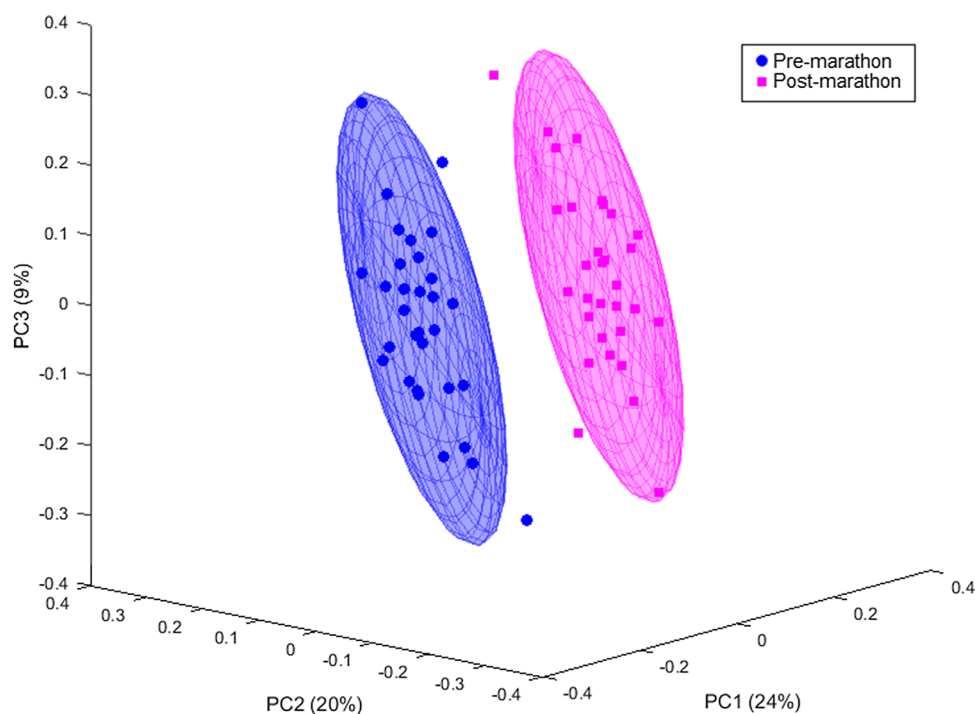


Table 2 The significant serum metabolite markers best describing the variation between the pre- and post-marathon groups, listed alphabetically

Metabolite name (PubChem ID)	Pre-marathon athletes Concentration (µg/mL) (standard deviation)	Post-marathon athletes Concentration (µg/mL) (standard deviation)	Wilcoxon signed rank test		PCA
			<i>p</i> value	<i>ω</i> value	
α-Aminomalonic acid (100714)	0.046 (0.022)	0.025 (0.009)	3.1×10^{-4}	0.458	0.031
α-Ethylhydracrylic acid (188979)	0.002 (0.001)	0.005 (0.002)	1.3×10^{-6}	0.615	0.025
α-Hydroxybutyric acid (11266)	0.019 (0.011)	0.045 (0.023)	2.8×10^{-6}	0.595	0.018
α-Hydroxyoctanoic acid (94180)	8.7×10^{-5} (5.3×10^{-5})	1.2×10^{-4} (6.3×10^{-5})	0.001	0.413	0.003
α-Ketoglutaric acid (51)	2.9×10^{-4} (2.1×10^{-4})	4.5×10^{-4} (1.8×10^{-4})	1.1×10^{-4}	0.490	0.009
α-Ketoisovaleric acid (5204641)	0.002 (0.001)	0.003 (0.001)	1.7×10^{-4}	0.478	0.017
α-Keto-β-methylvaleric acid (47)	0.009 (0.003)	0.007 (0.002)	0.005	0.358	0.025
α-Linolenic acid (5280934)	1.6×10^{-4} (8.1×10^{-5})	4.5×10^{-4} (2.6×10^{-4})	2.1×10^{-6}	0.602	0.021
β-Hydroxybutyric acid (441)	0.021 (0.019)	0.215 (0.161)	1.2×10^{-6}	0.617	0.030
β-Hydroxyhexanoic acid (151492)	3.2×10^{-4} (1.6×10^{-5})	8.9×10^{-4} (3.1×10^{-4})	2.1×10^{-6}	0.602	0.033
β-Hydroxyisobutyric acid (87)	2.9×10^{-5} (4.9×10^{-5})	2.9×10^{-4} (1.4×10^{-4})	1.2×10^{-6}	0.617	0.034
β-Hydroxyisovaleric acid (69362)	0.002 (0.001)	0.002 (0.001)	0.011	0.321	0.009
β-Hydroxypropionic acid (68152)	0.003 (0.001)	0.004 (0.001)	0.007	0.343	0.013
β-Hydroxy-α,β-didehydrosebacic acid (5366445)	0.005 (0.002)	0.006 (0.002)	0.009	0.333	0.013
5-Dodecenoic acid (5312377)	1.3×10^{-4} (2.8×10^{-5})	0.002 (0.001)	1.2×10^{-6}	0.617	0.037
5-Pregnene-3β,20α-diol (312224064)	3.0×10^{-4} (2.7×10^{-4})	6.1×10^{-4} (4.3×10^{-4})	3.4×10^{-6}	0.590	0.007
10-Heptadecenoic acid (86289714)	0.001 (0.001)	0.003 (0.001)	1.2×10^{-6}	0.617	0.030
11-Eicosenoic acid (142770)	0.001 (0.001)	0.004 (0.001)	1.3×10^{-6}	0.615	0.033
11,14-Eicosadienoic acid (3208)	0.002 (0.001)	0.003 (0.001)	9.3×10^{-4}	0.421	0.008
Acetoacetic acid (96)	7.5×10^{-5} (5.4×10^{-5})	2.1×10^{-4} (1.5×10^{-4})	0.002	0.393	0.014
Alanine (5950)	4.2×10^{-4} (2.9×10^{-4})	2.6×10^{-4} (1.5×10^{-4})	0.004	0.368	0.003
Arabitol (439255)	0.004 (0.001)	0.005 (0.002)	9.9×10^{-4}	0.418	0.006
Aspartic acid (5960)	0.020 (0.012)	0.013 (0.007)	0.014	0.311	0.019
Citric acid (311)	9.6×10^{-4} (4.5×10^{-4})	0.002 (0.001)	8.9×10^{-5}	0.498	0.012
Dimethylglycine (673)	0.023 (0.013)	0.017 (0.008)	0.011	0.324	0.008
D-Rhamnose (5460029)	0.001 (0.001)	0.001 (0.001)	1.1×10^{-2}	0.324	0.006
Erythritol (222285)	0.007 (0.002)	0.012 (0.004)	1.6×10^{-6}	0.610	0.016
Ethyl-α-D-glucopyranoside (91733361)	0.169 (0.085)	0.213 (0.094)	0.008	0.336	0.003
Fumaric acid (444972)	0.005 (0.002)	0.007 (0.002)	7.5×10^{-4}	0.428	0.018
Glucaric acid (33037)	4.1×10^{-4} (1.8×10^{-4})	0.001 (0.001)	0.002	0.391	0.010
Glucose (5793)	0.001 (0.001)	0.003 (0.005)	8.7×10^{-6}	0.565	0.025
Glutaric acid (743)	3.1×10^{-4} (1.9×10^{-4})	4.7×10^{-4} (2.8×10^{-4})	0.009	0.331	0.009
Glyceric acid (752)	0.005 (0.002)	0.008 (0.003)	6.9×10^{-5}	0.505	0.015
Glycerol (753)	0.088 (0.042)	0.447 (0.143)	1.2×10^{-6}	0.617	0.041
Glycine (750)	0.042 (0.019)	0.026 (0.013)	7.5×10^{-4}	0.428	0.024
Heptadecanoic acid (10465)	0.004 (0.001)	0.006 (0.001)	1.2×10^{-6}	0.617	0.020
Ibuprofen (3672)	7.5×10^{-6} (1.8×10^{-5})	0.004 (0.013)	6.4×10^{-5}	0.508	0.011
Indole-3-propionic acid (3744)	0.002 (0.001)	0.001 (0.001)	1.8×10^{-4}	0.475	0.004
Indole-3-acetic acid (802)	0.003 (0.002)	0.002 (0.001)	8.1×10^{-4}	0.426	0.021
Lauric acid (3893)	0.011 (0.003)	0.020 (0.005)	1.2×10^{-6}	0.617	0.026
Leucine (6106)	0.130 (0.087)	0.064 (0.044)	0.002	0.398	0.022
Linoleic acid (5280450)	0.124 (0.050)	0.168 (0.055)	4.9×10^{-4}	0.443	0.006
Malic acid (525)	0.004 (0.002)	0.007 (0.003)	3.1×10^{-6}	0.592	0.018
Malonic acid (867)	3.1×10^{-4} (1.2×10^{-4})	4.1×10^{-4} (1.7×10^{-4})	0.005	0.353	0.006
Mannitol (6251)	0.004 (0.004)	0.007 (0.010)	0.006	0.348	0.004
Mannose (18950)	3.2×10^{-4} (4.3×10^{-4})	0.003 (0.005)	5.5×10^{-6}	0.577	0.024
Methionine (6137)	0.006 (0.004)	0.003 (0.002)	6.9×10^{-4}	0.431	0.017
Myo-inositol (892)	0.031 (0.030)	0.045 (0.035)	8.1×10^{-4}	0.426	0.011

Table 2 (continued)

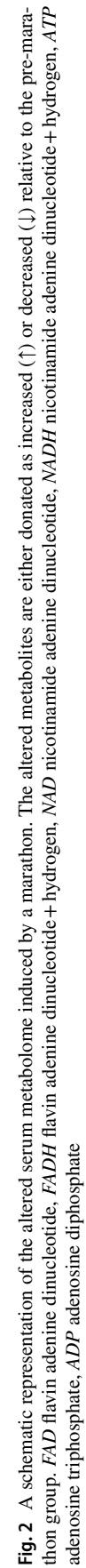
Metabolite name (PubChem ID)	Pre-marathon athletes	Post-marathon athletes	Wilcoxon signed rank test		PCA
	Concentration ($\mu\text{g/mL}$) (standard deviation)		<i>p</i> value	ω value	Power
Monopalmitin (14900)	3.9×10^{-4} (2.1×10^{-4})	6.7×10^{-4} (3.6×10^{-4})	0.002	0.401	0.011
Myristoleic acid (5281119)	0.001 (0.001)	0.006 (0.002)	1.2×10^{-6}	0.617	0.037
Oleic acid (445639)	0.127 (0.072)	0.511 (0.196)	1.3×10^{-6}	0.615	0.022
Palmitic acid (985)	0.423 (0.124)	0.633 (0.128)	1.6×10^{-5}	0.548	0.016
Palmitoleic acid (445638)	0.009 (0.008)	0.042 (0.022)	7.2×10^{-6}	0.570	0.026
Pentadecanoic acid (13849)	0.004 (0.002)	0.008 (0.002)	1.2×10^{-6}	0.617	0.022
Phenylalanine (6140)	0.030 (0.015)	0.020 (0.009)	0.002	0.393	0.021
<i>p</i> -Hydroxyphenylacetic acid (127)	0.002 (0.001)	0.003 (0.002)	0.001	0.408	0.008
<i>p</i> -Hydroxyphenyllactic acid (9378)	0.003 (0.002)	0.005 (0.002)	2.9×10^{-4}	0.460	0.014
Pyroglutamic acid (7405)	0.060 (0.014)	0.050 (0.023)	2.3×10^{-4}	0.468	0.009
Pyruvic acid (1060)	0.013 (0.009)	0.024 (0.012)	7.5×10^{-4}	0.428	0.016
Serine (5951)	0.033 (0.024)	0.018 (0.011)	0.014	0.314	0.021
Sorbose (439192)	0.015 (0.006)	0.022 (0.01)	6.1×10^{-4}	0.436	0.008
Squalene (638072)	0.002 (0.001)	0.003 (0.004)	0.006	0.351	0.011
Succinic acid (1110)	0.011 (0.008)	0.016 (0.010)	5.5×10^{-6}	0.577	0.006
Tagatofuranose (12306016)	0.101 (0.058)	0.190 (0.122)	3.9×10^{-4}	0.450	0.012
Talofuranose (15560229)	0.147 (0.050)	0.253 (0.090)	2.3×10^{-5}	0.538	0.011
Threonic acid (5460407)	0.011 (0.004)	0.016 (0.005)	1.1×10^{-5}	0.557	0.025
Threonine (6288)	0.021 (0.014)	0.011 (0.007)	0.003	0.381	0.017
Tridecanoic acid (12530)	2.4×10^{-4} (1.0×10^{-4})	3.8×10^{-4} (1.7×10^{-4})	9.5×10^{-6}	0.562	0.010
Tyrosine (6057)	0.019 (0.008)	0.014 (0.006)	0.007	0.343	0.018
Valine (6287)	0.188 (0.115)	0.094 (0.061)	9.3×10^{-4}	0.421	0.027

PCA principle component analysis

temporarily inactivate insulin-dependent glucose uptake systems (i.e. GLUT 4 transporters and glucokinase) (Salway 2012), preventing glucose absorption into cells. This could ascribe for the post-marathon elevations in serum glucose concentrations and various other associated metabolites, including glucaric acid (Żółtaszek et al. 2008) and mannose (Hu et al. 2016) as well as the elevated concentrations of gluconeogenesis-associated metabolites, i.e. myo-inositol (Eisenberg and Parthasarathy 1987), erythritol (synthesized via erythrose-4-phosphate in pentose phosphate pathway) (Hootman et al. 2017), glycerol and glyceric acid (Salway 2012; Wadman et al. 1976). Furthermore, the elevated concentrations of pyruvic acid observed in the post-marathon samples were anticipated since it is an end-product of the glycolysis pathway which feeds into the TCA cycle for further ATP production (Salway 2012). This is confirmed by the accumulation of various TCA cycle intermediates such as α -ketoglutaric acid, succinic acid, citric acid, fumaric acid and malic acid (Qiang 2015), which also indicate the accumulation of circulating NADH/FADH₂ molecules (Esterhuizen et al. 2017) as a result of a saturated ETC activity. It is also important to mention that many of the aforementioned carbohydrate metabolites, along with elevated concentrations of mannose (Hu et al. 2016), sorbose (Guzik and

Stachowicz 2016), mannitol (McNutt 2000), tagatofuranose (Kroger et al. 2006), and threonic acid (an ascorbic acid derivative) (Simpson and Ortwerth 2000), are well-known constituents of fruits/fruit juices, vegetables/vegetable juices, peanuts, energy bars, energy drinks and various other ergogenic aids consumed by athletes during the course of a marathon (Jeukendrup 2011; Pfeiffer et al. 2012).

The reduction in intracellular glucose due to the aforementioned cellular uptake inhibition is known to activate lipolysis of free and adipose tissue-bound triacylglycerol (TAG) (MacLaren and Morton 2012) as alternative fuel substrates. This is substantiated by the elevated concentrations of serum glycerol, monopalmitin and various free fatty acids (lauric acid, palmitic acid, palmitoleic acid, 11-eicosenoic acid, 11,14-eicosadienoic acid, myristoleic acid, α -linolenic acid, 5-dodecenoic acid, linoleic acid and oleic acid) (Kujala et al. 2013; Lewis et al. 2010; Peake et al. 2014; Waśkiewicz et al. 2012). Additionally, accumulated 3-hydroxy acids (β -hydroxyhexanoic acid) and 3-keto acids (β -hydroxy- α,β -didehydrosebacic acid) are indications of a saturated β -oxidation pathway, ascribed to the inhibition of the rate-limiting enzyme, β -hydroxyacyl dehydrogenase, which is pursued by 3-ketoacyl-CoA thiolase. This saturated β -oxidation pathway results in the



catabolism of the accumulated fatty acids via α -oxidation (Roe and Ding 2001), thus substantiating the elevated concentrations of α -hydroxyoctanoic acid (C_8 ; an α -oxidation intermediate) and the odd-chain fatty acids (OCFA; tridecanoic acid [C_{13}], pentadecanoic acid [C_{15}], heptadecanoic acid [C_{17}] and 10-heptadecenoic acid [$C_{17:1}$]) detected in the post-marathon serum. It should, however, be mentioned that these OCFA may also arise from elevated dietary intake (Jenkins et al. 2017) and/or autophagy of various cellular constituents during extensive energy-requiring states (Singh and Cuervo 2011). Nevertheless, irrespective of their origins, these OCFAs are ultimately catabolized to propionyl-CoA (Pfeuffer and Jaudszus 2016), hence the elevated β -hydroxypropionic acid observed in the post-marathon serum. The elevated concentrations of β -hydroxybutyric acid and acetoacetic acid are anticipated, as these are alternative fuel substrates for the brain (Cahill and Vech 2003) and skeletal muscles (Holloszy and Coyle 1984) in hypoglycemic states, and could also be an indication of an imbalanced redox state (Esterhuizen et al. 2017; Salway 2012). Furthermore, the post-marathon elevations of malonic acid typically indicate the accumulation of malonyl-CoA, which is a long-chain fatty acid (LCFA) transport inhibitor (Salway 2012) and could therefore be an additional reason for the increased cytosolic LCFAs (palmitic acid, palmitoleic acid, 11-eicosenoic acid, 11,14-eicosadienoic acid, myristoleic acid, α -linolenic acid, linoleic acid and oleic acid).

Most amino acids are catabolized into TCA cycle substrates via propionyl-CoA, succinyl-CoA, pyruvic acid or acetyl-CoA, depending on the specific amino acid (Salway 2012). Reduced concentrations of amino acids (serine, glycine, alanine, aspartic acid, phenylalanine, tyrosine, threonine and methionine) and altered amino acid-associated metabolite concentrations [dimethylglycine (Holm et al. 2003), pyroglutamic acid (Kumar and Bachhawat 2012), indole-3-acetic acid (Salway 2012) and glutaric acid (Peake 2016)] were detected in the post-marathon serum, which indicate amino acid catabolism during the marathon. Furthermore, elevated concentrations of α -hydroxybutyric acid (an intermediate in the threonine/methionine pathway), further supports the aforementioned NADH:NAD⁺ imbalance, ketoacidosis, reduced insulin secretion and impaired glucose absorption (Gall et al. 2010). Additionally, elevated *p*-hydroxyphenylacetic acid and *p*-hydroxyphenyllactic acid could be indicative of mild liver injury/damage (Ghoraba et al. 2014; Liebich and Pickert 1985), which concur with previous findings (Jastrzebski et al. 2015; Lippi et al. 2011).

In accordance with previous findings (Pechlivanis et al. 2010), reduced serum concentrations of the branched-chain amino acids (BCAAs) isoleucine (0.05 vs 0.02 $\mu\text{g/mL}$, $p=0.018$), valine and leucine were detected in the post-marathon samples, while their catabolism intermediates i.e. α -ketoisovaleric acid, β -hydroxyisobutyric acid (valine

metabolites) and α -ethylhydracrylic acid (allo-isoleucine metabolism) (Wendel et al. 1989), were significantly elevated. Allo-isoleucine catabolism intermediates may serve as alternative substrates in the valine catabolism pathway (Ryan 2015), however, since the valine catabolism intermediates remained elevated in the post-marathon serum it can be deduced that allo-isoleucine is instead catabolized to α -ethylhydracrylic acid (Korman et al. 2005) via α -keto- β -methylvaleric acid (explaining its reduced concentrations post-marathon) with the subsequent production of FADH₂ (Korman et al. 2005). The elevated concentrations of β -hydroxyisovaleric acid detected post-marathon is typically associated with ketone rich environments (as observed in the current investigation), resulting from increased leucine catabolism and the subsequent production of isovaleryl-CoA`23 (Mock et al. 2011). This metabolite is also a well-known constituent of athlete supplementation (Brioche et al. 2016) and could be elevated due to dietary ingestion during the marathon. Furthermore, reduced concentrations of these BCAAs, in particular leucine, results in mammalian target of rapamycin complex 1 (mTOR1) inhibition (Laplane and Sabatini 2009), which in turn activates various catabolic processes such as autophagy (of organelle and plasma membrane constituents) to release additional embedded fuel substrates (Singh and Cuervo 2011). mTOR1 inhibition can also be induced by other factors including elevated 5'-AMP-activated protein kinase during energy deprivation, reduced oxygen levels, reduced essential amino acids and inflammation (Laplane and Sabatini 2009), all of which are associated with endurance races and evidently occur in the marathon athletes investigated in this study. Considering that autophagy contributes to elevated amounts of cellular debris, the aforementioned accumulation of fatty acids (especially the OCFAs) in the post-marathon serum may also be explained by the autophagosomal degradation of phospholipids, sphingolipids (Kishimoto et al. 1973; Maes et al. 1996) and phytosphingosines (Kitamura et al. 2017; Kondo et al. 2014) found in cell and organelle membranes. Additionally, the elevated concentrations of squalene and 5-pregnene-3 β ,20 α -diol in the post-marathon serum suggest cholesterol degradation (Charlton-Menys and Durrington 2007; Salway 2012), further supporting the activation of autophagy as the latter is a common constituent in cell membranes (Salway 2012). Elevated 5-pregnene-3 β ,20 α -diol (also known as 20 α -dihydropregnenolone) post-marathon also indicates steroid metabolism activation via cholesterol catabolism as it is produced by the reduction of pregnenolone via 20-hydroxysteroid dehydrogenase (Ebner et al. 2006). Pregnenolone is a precursor for the production of aldosterone and cortisol, the latter of which stimulates lipolysis, gluconeogenesis and protein catabolism (Salway 2012).

Furthermore, a significant decrease in α -aminomalonic acid concentrations was observed in the post-marathon

serum samples. Although the precise origin of this metabolite is unknown, it has been associated with abnormal protein oxidation, macrophage accumulation, non-essential amino acid oxidation via reactive oxygen species and pulmonary arterial hypertension (Bujak et al. 2016).

The microbiome plays a crucial role in many essential metabolic processes required during strenuous exercise, including the regulation of energy metabolism, oxidative stress and inflammatory response pathways (Mach and Fuster-Botella 2017). To this end, various microbial-associated metabolites (i.e. tagatofuranose, talofuranose, ethyl- α -D-glucopyranoside, arabinol, indole-3-propionic acid and D-rhamnose) were significantly altered in the post-marathon samples. Most of these metabolites are synthesized from host carbohydrate intermediates, either via catabolism or fermentation processes [arabinol: a sugar alcohol produced from arabinose/glucose (Kumdam et al. 2014); and ethyl- α -D-glucopyranoside: an ethanolysis product from glucose (Hu et al. 2013)]. Indole-3-propionic acid is the deamination product of tryptophan, produced by gut microbes (e.g. *Clostridium sporogenes*) (Wikoff et al. 2009) and further supports the notion of reduced amino acids. The reduced concentrations of D-rhamnose, a component of most Gram-positive bacterial cell walls (Mistou et al. 2016), contradict the above-mentioned microbial product elevations. Since the particular nature of this reduction following completion of the marathon is unclear, it warrants future investigation.

In addition to these endogenously and microbially produced metabolites, elevated concentrations of ibuprofen were also observed post-marathon. Ibuprofen is a well-known non-steroidal, anti-inflammatory drug (Nieman et al. 2006) commonly used by athletes for preventing muscle damage/soreness and inflammation and was most likely consumed by some athletes prior to/during the marathon (Meanulty et al. 2007).

5 Conclusion

The results of this metabolomics investigation suggest that the body utilizes various fuel substrate pathways to comply with the high energy demands required during the marathon, including catabolism of carbohydrates, lipids (β -oxidation and α -oxidation) and amino acids, as well as the activation of ketogenesis and autophagy via mTOR1 inhibition. Considering the results of the current investigation and previous literature, the possible cascade of events contributing to this metabolic “snapshot” could be summarized as follows: (a) A proposed initial reduction in carbohydrate catabolism and glucose uptake via insulin-dependent transporters lead to glycolysis dysregulation, ketogenesis activation and increased serum glucose. (b) A metabolic shift towards fatty acid utilization (from either endogenous or

dietary TAGs) is induced, which (c) overwhelms/saturates the β -oxidation pathway, resulting in the α -oxidation of fatty acids. (d) Amino acids (from either endogenous or dietary protein catabolism) are also used as alternative fuel substrates, resulting in (e) mTOR1 inhibition and autophagy as the body desperately tries to generate the necessary fuel substrates to comply with the energy demand. (f) Lastly, various metabolic processes are activated to reduce oxidative stress and regulate/correct the redox imbalance.

Possible limitations of this study include human genotype/phenotype variation (an inevitable confounder) and the uncontrolled dietary intake of the athletes during the marathon. However, convincing athletes to deviate from their individualized supplementation protocols would be extremely difficult, if not impossible. Validation using a larger sample cohort could further substantiate the current findings. Nonetheless, these findings indicate the extensive metabolic changes induced by the marathon perturbation. Possible future prospects could be to investigate the effects of supplementing with amino acids, pre- and/or pro-biotics, and β -hydroxyisovaleric acid as a means of improving aerobic exercise performance, reduce skeletal muscle/liver damage, and enhance recovery.

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Author contributions The concept and study were designed by DTL, ZS, GH, TC, KMK and EJS; samples were acquired from the Northumbria University in collaboration with GH, TC, KMK and EMS. ZS was responsible for manuscript drafting, data analysis and interpretation, the latter of which was assisted by DTL, LL and LJM. LL, LJM and DTL were involved in repeated manuscript reviewing, of which LL was greatly involved with structural (format) editing. All of the authors revised and approved the final version of this manuscript.

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Data availability The current analysis is part of a larger study consisting of multiple aims which are being drafted into various manuscripts. Considering this, the datasets generated from this investigation are not publically available, but can be acquired from the corresponding author on reasonable request. The authors declare that all the results included within this study have been presented clearly, honestly and without fabrication, falsification, or inappropriate data manipulation.

Compliance with ethical standards

Conflict of interest The authors declare that there are no conflicts of interest, and that this manuscript, and the work described therein, is unpublished and has not been submitted for publication elsewhere.

Ethical approval Ethical approval for this investigation, conducted according to the Declaration of Helsinki and International Conference on Harmonization Guidelines, was obtained from the Research Ethics Committee of the Faculty of Health and Life Sciences at the North-

umbria University in Newcastle upon Tyne, UK (Reference Number: HLSTC120716).

Informed consent Informed consent was obtained from all individuals included in the study.

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The unaided recovery of marathon-induced serum metabolome alterations

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Endurance athlete performance is greatly dependent on sufficient post-race system recovery, as endurance races have substantial physiological, immunological and metabolic effects on these athletes. To date, the effects of numerous recovery modalities have been investigated, however, very limited literature exists pertaining to metabolic recovery of athletes after endurance races without the utilisation of recovery modalities. As such, this investigation is aimed at identifying the metabolic recovery trend of athletes within 48 h after a marathon. Serum samples of 16 athletes collected 24 h before, immediately after, as well as 24 h and 48 h post-marathon were analysed using an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry metabolomics approach. The metabolic profiles of these comparative time-points indicated a metabolic shift from the overall post-marathon perturbed state back to the pre-marathon metabolic state during the recovery period. Statistical analyses of the data identified 61 significantly altered metabolites including amino acids, fatty acids, tricarboxylic acid cycle, carbohydrates and associated intermediates. These intermediates recovered to pre-marathon related concentrations within 24 h post-marathon, except for xylose which only recovered within 48 h. Furthermore, fluctuations in cholesterol and pyrimidine intermediates indicated the activation of alternative recovery mechanisms. Metabolic recovery of the athletes was attained within 48 h post-marathon, most likely due to reduced need for fuel substrate catabolism. This may result in the activation of glycogenesis, uridine-dependent nucleotide synthesis, protein synthesis, and the inactivation of cellular autophagy. These results may be beneficial in identifying more efficient, targeted recovery approaches to improve athletic performance.

Endurance races (> 5 km) have become increasingly popular over the last decade and primarily include half-marathons (21.1 km), marathons (42.2 km), and ultra-marathons (> 42.2 km)¹. Participation in these events requires extensive preparation including physical and mental conditioning as well as meticulously planned dietary strategies. According to Barnett², optimal athletic performance is only achieved when these principles are accompanied by sufficient system recovery following any endurance activity. “Recovery” comprehensively refers to the process in which an altered biological system, such as the metabolome, reverts to its corresponding pre-perturbed state³. In endurance athletes, metabolic recovery is thought to proceed in a biphasic manner with the initial phase mainly consisting of rapid oxygen, ATP and phosphocreatine replenishment, whereas the second phase entails the slow restoration of innate metabolism adaptations^{2,3}. A popular research approach for identifying and mapping such metabolic adaptations, quantitatively and qualitatively, is collectively referred to as “metabolomics”. Considering that the metabolome provides a direct depiction of the physiological state of an organism at a specific point in time⁴, metabolomics is a formidable tool when investigating the effects of endurance races on the human body, as well as the recovery thereof.

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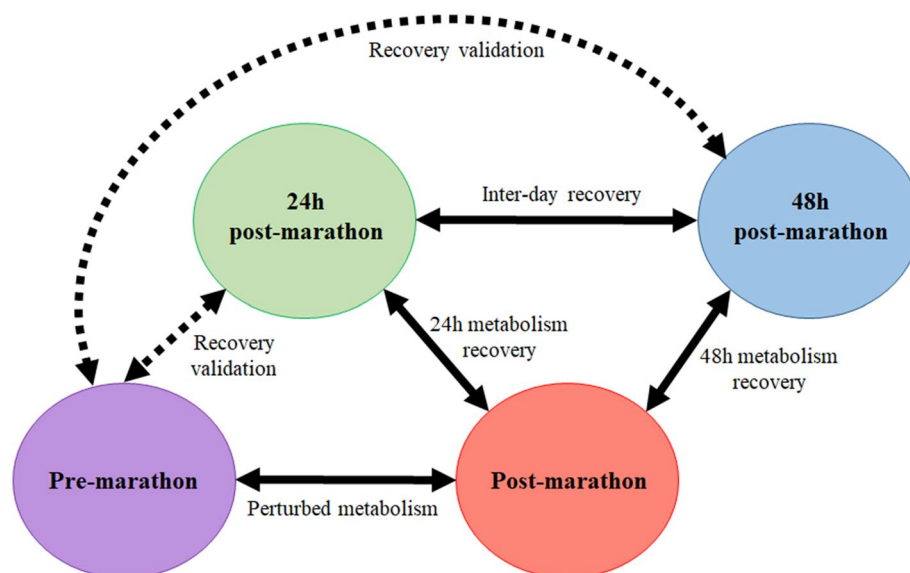


Figure 1. Schematic representation of the sample group comparisons performed during this investigation.

Previous studies investigating the perturbed metabolism of endurance athletes mainly observed elevated concentrations of carbohydrates, fatty acids, and ketones, accompanied by reduced concentrations of amino acids⁵. This is proposed to result from the dramatic metabolic shift between numerous fuel substrate catabolism systems, as well as alternative energy-producing mechanisms (α -oxidation and autophagy) activated during strenuous activity^{6–10}. Additionally, the extensive reduction in fuel substrates was associated with alterations in the urea cycle, purine, steroid and pyrimidine metabolism, reactive oxygen species production pathways^{5,6,9–13}, as well as the gut microbiome^{9,14}.

Given the nature and extent of the physiological, immunological and metabolic effects of endurance exercise on the human body, a substantial amount of research has been directed towards investigating various therapeutic recovery modalities including cryotherapy, massage, heat therapy, stretching, compression, and non-steroidal anti-inflammatory drugs^{2,15–17}, as well as the ingestion of functional foods, such as cherries, pomegranates, and green leafy vegetables¹⁸. However, very limited literature is available regarding the overall recovery of the perturbed metabolism after an endurance race, without the ingestion and/or utilisation of recovery modalities. From these studies, it is evident that during the immediate hours after recovery, myocellular glycogen restoration^{19,20}, as well as phosphocreatine store replenishment and blood lactate removal takes preference^{2,3,21}, while lipids are temporarily further oxidised as an energy source^{19,20}. Considering this, it is suggested that glycogen restoration is attained within 24–48 h after endurance exercise²⁰, whereas amino acids, fatty acids, fatty acid-carnitine conjugates along with tricarboxylic acid (TCA) cycle and pyrimidine/purine pathway intermediates remain perturbed for at least 14 h after endurance running^{5–7,9}.

Considering the current lack of literature regarding the metabolic recovery of marathon athletes over an extended period (48 h), an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry (GCxGC-TOFMS) serum metabolomics approach was used to investigate the unaided (without the ingestion and/or utilisation of recovery modalities) metabolic recovery (to baseline-related concentrations) of 16 marathon athletes within 48 h post-marathon. These results might improve the current knowledge pertaining to the consequence of endurance activity and the time-course of recovery in the days following the event, as well as lead to the identification of new or additional recovery pathways and/or more targeted, perhaps even personalised, recovery approaches.

Results

The GCxGC-TOFMS system detected a total of 1,308 peaks of which 444 peaks were classified as “Analytes/Unknowns”. After removing column and/or reagent-related compounds, as well as following the merging of duplicate peaks of compounds, such as sugars and amino acids, 839 compounds (including the 444 peaks classified as “Analytes/Unknowns” and internal standard) remained. The entire list of 838 compounds (excluding internal standard) was then subjected to the aforementioned processing steps [i.e. quality control coefficient of variation (QC-CV) filter etc.] and further statistical analyses. Considering the aim of this investigation, multiple group comparisons were required (Fig. 1), subsequently leading to numerous statistical outputs, which will be discussed below.

Multivariate statistical output. In order to visualise the overall metabolic shift over the entire time-frame of the study, an analysis of variance (ANOVA) simultaneous component analysis (ASCA) model was constructed with the resulting scores graphically displayed and grouped according to the time component of the current study design (Fig. 2). All scripts used here are readily available in the mentioned toolboxes provided by

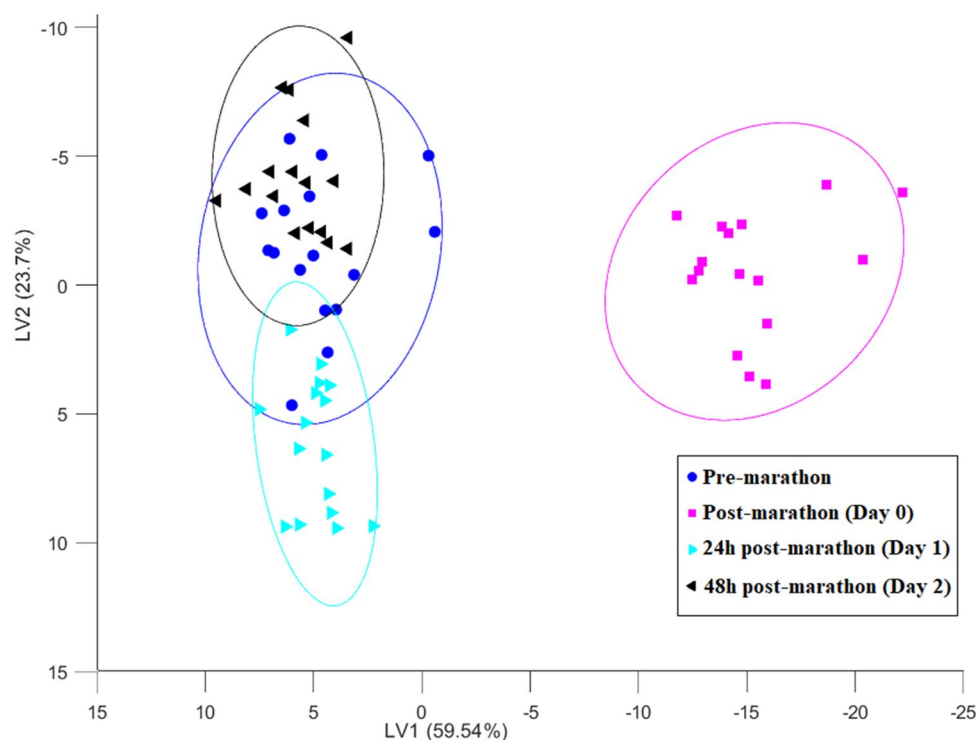


Figure 2. The ASCA plot indicating the natural, time-dependent differentiation of the comparative groups: pre-marathon (denoted by blue/circle), post-marathon (denoted by pink/square), 24 h post-marathon (denoted by turquoise/right-tilted triangle) and 48 h post-marathon (denoted with black/left-tilted triangle). The variance accounted for by each latent variable (LV) is indicated in parenthesis on the respective axes. The ellipsoids represent 95% confidence intervals of each time-points centroid.

Eigenvector Research Inc., with the exception of the ASCA scripts, which can be downloaded from <https://www.bdagroup.nl/content/Downloads/software/software.php>. Based on Fig. 2, it is evident that although a marathon has a dramatic impact on the human serum metabolome these changes recover within 48 h, as indicated by the confined positioning of the 24 h and 48 h post-marathon ellipsoids relative to that of the pre-marathon. Furthermore, in an attempt to inspect specific adaptations between time-points, individual multi-level principal component analysis (ML-PCA) plots for the specified group comparisons (Fig. 1) are presented in Supplementary Fig. S1a–f. The following can be deduced from these figures (S1a–f): (1) As expected, the marathon perturbation (Fig. S1a) induced significant metabolic alterations, which is supported by Stander et al.¹⁰; (2) Clear separation (no overlap) of the recovery time-points (24 h and 48 h post-marathon) and post-marathon (day 0) ellipsoids (Fig. S1b and c) indicate a significant deviation from the post-marathon perturbed metabolic state; (3) The magnitude of metabolome variation presented 24 h and 48 h post-marathon (Fig. S1 f) seems rather minor as these ellipsoids appear relatively grouped (ellipsoid overlap); (4) The confined positioning of the 24 h and 48 h post-marathon ellipsoids relative to the pre-marathon (Fig. S1 d and e) indicate the recovery of the metabolome to a baseline-related state (overlapping ellipsoids). Furthermore, in order to visualise metabolite stratification among the comparative groups, clustering dendrograms were constructed and are included in the supplementary material (Fig. S3). From these figures, it is clear that stratification associations did not correlate with the comparative groups.

However, considering the relatively small sample set and the likelihood of increased false discovery rates (FDR; high dimensionality), along with controlling for model overfitting, multivariate models could not be validated and were therefore not used for variable selection but rather for global metabolic shift visualisation.

Univariate statistical outputs. Considering the aforementioned, variable selection was based on univariate comparisons as a means of determining the specific metabolic adaptations apparent during the course of this investigation while controlling the FDRs and avoiding overfitting. Although repeated measures ANOVA models may have been appropriate, paired t-tests were preferred since the dramatic effect of the marathon would result in a large number of significant metabolites requiring pairwise comparisons as post-hoc tests in any event. Based on this, only metabolites with a Benjamini–Hochberg p value ≤ 0.05 and an effect size d -value ≥ 0.5 (moderate effect) in each group comparison (Fig. 1), were deemed to be statistically significant. This approach yielded a list of 74 metabolite markers, of which 61 were positively annotated (the remaining 13 metabolites were classified as “Unknowns/Analytes”) and are listed in Table 1. A more comprehensive table including average concentrations at each of the comparative time-points has been included in Supplementary Table S1.

Compound name (PubChem ID)	PM/PrM ratio	D1/PM ratio	D2/PM ratio	D2/D1 ratio	D1/PrM ratio	D2/PrM ratio
Carbohydrate and associated intermediates						
Arabitol (439255)	1.36*	0.75*	0.65*	0.87	1.01	0.88
Erythritol (222285)	1.77*	0.61*	0.39*	0.63	1.08	0.68
Glucose (5793)	8.91*	0.17*	0.09*	0.52	1.48	0.77
Mannitol (6251)	1.34	0.78	0.58*	0.75	1.05	0.78
Mannose (18950)	15.72*	0.11*	0.05*	0.49	1.66	0.82
Myo-inositol (440194)	1.10	0.76*	0.63*	0.82	0.84	0.69
Psicose (441036)	1.15	0.45*	0.70	1.56	0.52	0.80
Rhamnose (5460029)	0.62*	1.51	1.86*	1.23	0.94	1.16
Ribose (10975657)	1.16	0.58*	0.89	1.53	0.67	1.03
Sorbose (439192)	1.46	0.69	0.64*	0.94	1.01	0.94
Tagatose (439312)	1.87*	0.56*	0.53*	0.95	1.04	0.99
Talose (441035)	1.69*	0.57*	0.52*	0.91	0.96	0.87
Threonic acid (151152)	1.39*	0.80*	0.78*	0.98	1.11	1.09
Uridine (6029)	1.06	0.61*	1.02	1.66*	0.65	1.08
Xylose (135191)	0.90	0.52*	0.76	1.45	0.47*	0.69
Lipid and associated intermediates						
α -Hydroxyoctanoic acid (94180)	1.49*	0.63*	0.67	1.06	0.94	0.99
α -Linolenic acid (5280934)	1.26*	0.69*	0.74*	1.07	0.87	0.93
β -Hydroxyhexanoic acid (11829482)	3.00*	0.38*	0.31*	0.82	1.13	0.92
5-Dodecenoic acid (5312377)	35.22*	0.02*	0.06*	3.73	0.59	2.19
10-Heptadecenoic acid (5312434)	5.87*	0.12*	0.15*	1.22	0.73	0.89
11,14-Eicosadienoic acid (5282805)	1.94*	0.54*	0.45*	0.83	1.05	0.87
11-Eicosenoic acid (5282768)	3.48*	0.31*	0.37*	1.20	1.06	1.28
Docosahexaenoic acid (445580)	1.17	0.69*	0.80	1.17	0.80	0.94
Eicosapentaenoic acid (446284)	1.30	0.69*	0.74*	1.07	0.90	0.96
Glycerol (753)	5.84*	0.17*	0.19*	1.11	0.99	1.10
Glycerol monopalmitic acid (308463)	1.87*	0.44*	0.55*	1.24	0.82	1.02
Heptadecanoic acid (10465)	1.82*	0.52*	0.52*	1.01	0.94	0.95
Lauric acid (3893)	2.07*	0.57*	0.46*	0.81	1.17	0.95
Methyl oleic acid (9922235)	1.68*	0.48*	0.72	1.51	0.81	1.22
Myristoleic acid (5281119)	12.33*	0.06*	0.08*	1.32	0.70	0.93
Oleic acid (445639)	5.04*	0.21*	0.22*	1.07	1.06	1.13
Palmitic acid (985)	1.67*	0.55*	0.57*	1.04	0.93	0.96
Palmitoleic acid (445638)	6.26*	0.19*	0.22	1.17	1.20	1.40
Pentadecanoic acid (13849)	2.21*	0.51*	0.43*	0.85	1.13	0.96
Ketones and TCA cycle intermediates						
α -Ketoglutaric acid (51)	1.57*	0.58*	0.64*	1.11	0.91	1.01
β -Hydroxybutyric acid (441)	12.98*	0.06*	0.06*	1.12	0.72	0.81
Acetoacetic acid (96)	2.89	0.37*	0.19*	0.51	1.07	0.54
Citric acid (311)	1.46	0.74	0.58*	0.78	1.08	0.85
Fumaric acid (444972)	1.42	0.75	0.68*	0.91	1.06	0.96
Malic acid (525)	1.62*	0.57*	0.52*	0.90	0.93	0.84
Malonic acid (867)	1.37	0.72	0.62*	0.86	0.99	0.85
Amino acid and cholesterol-associated intermediates						
α -Ethylhydracrylic acid (188979)	2.24*	0.48*	0.47*	0.97	1.08	1.05
α -Hydroxybutyric acid (11266)	2.53*	0.52*	0.49*	0.93	1.31	1.23
β -Hydroxyisobutyric acid (87)	15.39*	0.04*	0.06*	1.29	0.67	0.87
β -Hydroxyisovaleric acid (69362)	1.15	0.89	0.75*	0.84	1.02	0.86
5-Pregnen-3 β ,20 α -diol (312224064)	1.93*	0.38*	0.36*	0.96	0.73	0.70
Alanine (5950)	0.64	2.34*	1.86*	0.79	1.51	1.19
Aminomalonic acid (100714)	0.48	2.10*	2.06*	0.98	1.01	0.99
Aspartic acid (5960)	0.60	2.03*	1.36	0.67	1.22	0.82
Glutamic acid (33032)	0.79	1.51*	1.39	0.92	1.19	1.10
Glutaric acid (743)	1.73	1.12	0.52*	0.46	1.94	0.89
Continued						

Compound name (PubChem ID)	PM/PrM ratio	D1/PM ratio	D2/PM ratio	D2/D1 ratio	D1/PrM ratio	D2/PrM ratio
Glycine (750)	0.59	1.79*	1.45	0.81	1.06	0.86
Hydroxyproline (5810)	0.44	2.03	2.68*	1.32	0.89	1.18
Methionine (6137)	0.54	2.17*	1.88*	0.87	1.18	1.02
Phenylalanine (6140)	0.64	1.93*	1.47*	0.76	1.24	0.95
<i>p</i> -Hydroxyphenylacetic acid (127)	2.00	0.51*	0.45*	0.88	1.03	0.90
<i>p</i> -Hydroxyphenyllactic acid (9378)	1.53	0.66*	0.58*	0.88	1.01	0.89
Pyroglutamic acid (7405)	0.71*	1.35*	1.41*	1.04	0.95	1.00
Squalene (638072)	3.39*	0.31*	0.34*	1.07	1.06	1.14
Tyrosine (6057)	0.64	1.78*	1.57*	0.88	1.14	1.01
Valine (6287)	0.53	1.83	2.21*	1.21	0.97	1.18

Table 1. Ratio of concentration fluctuations in the serum metabolite markers (n = 61) best describing the detected variation and metabolic recovery of the athletes within 48 h post-marathon. *PrM* Pre-marathon, *PM* Post-marathon, *D1* 24 h post-marathon, *D2* 48 h post-marathon. Significant *p*- and *d*-values (*).

Discussion

Table 1 depicts the recovery trend of the metabolites not only initially affected by the marathon perturbation, but also the concentration fluctuations of additional metabolites pertinent to the 24 h and 48 h recovery period. In order to ascertain the metabolic recovery mechanisms following a marathon, it is imperative to first consider the immediate effects of a marathon on the serum metabolome of athletes, which was characterised and previously described by Stander et al.¹⁰. Briefly, alterations were mainly observed in metabolic pathways associated with energy production, such as elevated concentrations of various carbohydrates, TCA cycle intermediates, fatty acids and ketone bodies, and reduced concentrations of various amino acids and associated metabolites. The perturbed metabolic state may also be linked to imbalanced redox potential, the saturation of traditional fatty acid catabolic pathways, possible insulin secretion hampering, and the activation of cellular autophagy to provide additional fuel substrates.

A detailed description of the recovery (to pre-marathon related concentrations) of the identified metabolites are discussed in a grouped-manner, based on metabolic alterations of the recovery time-points (24 h and 48 h post-marathon; day 1 and day 2) relative to the perturbed metabolic state (day 0; immediately post-marathon) and is schematically summarised in Fig. 3.

Carbohydrate metabolism. Carbohydrates, glucose in particular, are considered to be the primary fuel substrates used by the body during physical activity. Stander et al.¹⁰ and Lewis et al.⁶ reported severely elevated serum concentrations of various carbohydrates immediately after a marathon (day 0). In this metabolomics investigation, it is clear that the majority of the serum carbohydrates (glucose and mannose) and associated metabolites (uridine, ribose, glycerol, and myo-inositol) returned to baseline (pre-marathon) concentrations within 24 h post-marathon (Table 1), with the exception of xylose (also dietary-related). This can be ascribed to the restoration of the glycogen stores, which were perceived as "depleted" in the initial stages of the marathon¹⁰.

Moreover, substantial reductions in uridine concentrations were observed within 24 h post-marathon (Table 1). Uridine is a nucleoside formed via the conjugation product of ribose and uracil and is a key component in RNA and subsequently protein synthesis²². This, in conjunction with the reduced ribose, indicated possible increased nucleotide production via the activation of the pyrimidine salvage metabolism. The pyrimidine salvage metabolism refers to the adaptive mechanism by which nucleotides can be synthesised using pre-formed nucleosides and nucleobases, usually following a nucleotide degradation period^{5,22}, such as the extensive protein catabolism induced by a marathon^{6,7,10}. These recycled nucleotides can also interact with lipids to form pyrimidine nucleotide–lipid conjugates, a key component in cell membrane structures²². Considering this, the slight reduction in uridine concentrations observed 24 h after the completion of the marathon, may also be associated with the restoration of damaged cellular membranes, which occur as a result of strenuous exercise¹⁰. This is further substantiated by the reduction/recovery of myo-inositol concentrations within 24 h post-marathon, as it is a well-known constituent of glycerophospholipids²³.

Additionally, the de novo pyrimidine synthesis pathway is dependent on various elements of the pentose phosphate pathway²², particularly ribose-5-phosphate and phosphoribosyl pyrophosphate²⁴. Since these are interlinking substrates for the production of erythrose-4-phosphate²⁴, the recovery of erythritol (produced from the latter)²⁵ not only suggests the activation of glycogenesis via the utilisation of pentose phosphate pathway intermediates, but further supports the activation of the abovementioned nucleotide repair mechanisms. Furthermore, since the majority of the erythritol is absorbed rather than metabolized²⁶, as well as the fact that it is a well-known alternative sugar added to low-calorie foods²⁷ frequently ingested by athletes, the alterations of this metabolite 24 h and 48 h post-marathon may also be due to reduced ingestion of such foods by the athletes during the recovery period. Similarly, several of the metabolites in Table 1, including mannitol, psicose, sorbose^{27,28}, tagatose²⁹, and xylose²⁴, are natural constituents of fruit and vegetables, Food and Drug Administration approved alternative bulk sweeteners, and/or the products of metabolised sweeteners such as threonic acid³⁰, and arabinol via arabinose³¹. Considering that endurance races reportedly alter gut microbiome composition, as well as increase intestinal permeability resulting in gut microbiome (GM) metabolite migration³², reductions in several

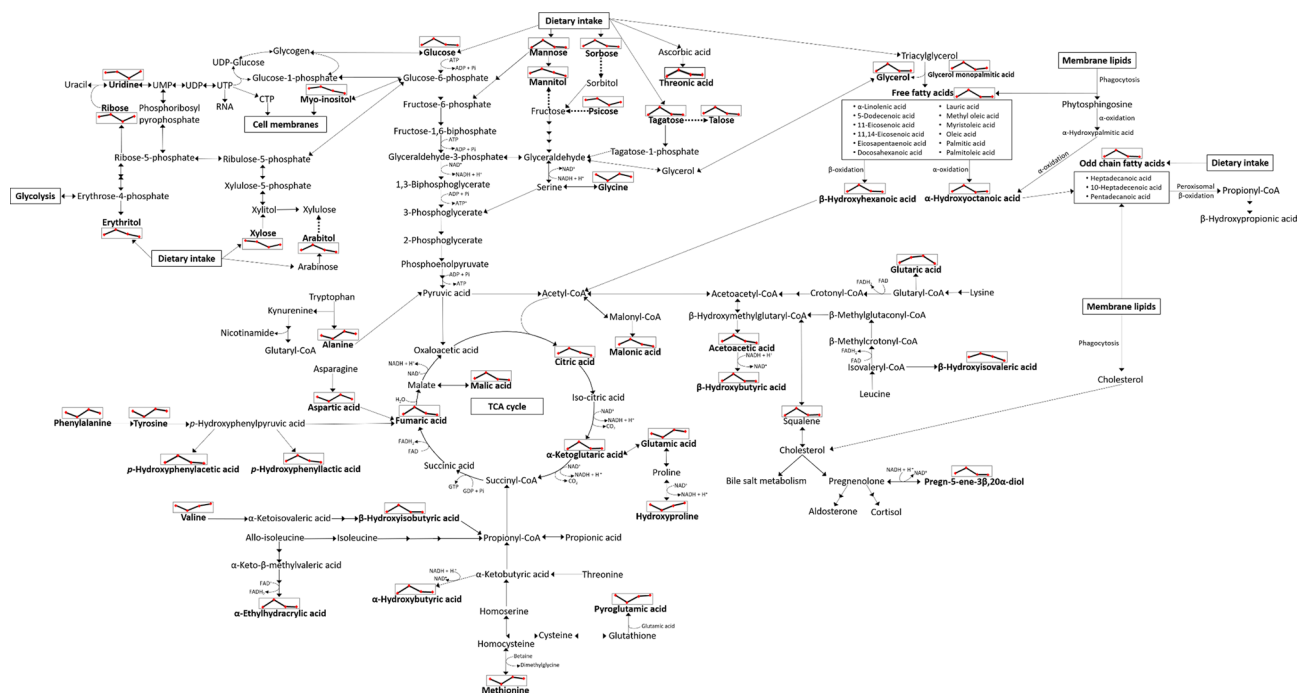


Figure 3. A metabolic chart summarising the major pathways affected during and after a marathon perturbation. Microbial interactions are denoted with bold dashed arrows and the line-graphs schematically illustrate the time-dependant concentration shifts of the significantly altered metabolites. CoA coenzyme A, CO₂ carbon dioxide, CTP cytidine triphosphate, FAD⁺ flavin adenine dinucleotide, FADH₂ flavin adenine dinucleotide + hydrogen, GDP guanosine diphosphate, GTP guanosine triphosphate, NAD⁺ nicotinamide adenine dinucleotide, NADH nicotinamide adenine dinucleotide + hydrogen, Pi inorganic phosphate, TCA tricarboxylic acid, RNA ribonucleic acid, UDP uridine diphosphate, UMP uridine monophosphate, UTP uridine triphosphate (adapted from Stander et al.¹⁰).

of the detected metabolite markers (Table 1)^{31,33} could also be ascribed to the restoration of GM homeostasis and a reduction in host substrate availability/dietary ingestion.

Lipid metabolism. The aforementioned depletion of intracellular glucose and glycogen stores results in extensive lipolysis of triacylglycerols in adipose tissue, skeletal muscle and the liver during strenuous activity^{5,10,34}. Reduced lipolysis intermediate (α -linolenic acid, 11,14-eicosadienoic acid, 11-eicosenoic acid, 5-dodecenoic acid, docosahexaenoic acid, eicosapentaenoic acid, lauric acid, myristoleic acid, oleic acid, palmitic acid, palmitoleic acid, glycerol, and glycerol monopalmitic acid) concentrations were observed within 24 h post-marathon (Table 1) and coincided with similar findings from previous literature^{5–7}. The normalisation of these is a clear attestation of the down-regulation of lipolysis⁷ mainly due to the aforementioned restoration of the glycaemic flux, concurrent with reduced ATP requirements during passive recovery^{34,35}. The initial rapid reduction in serum fatty acids within 24 h post-marathon followed by a slight increase after 48 h post-marathon (Table 1) suggests a temporary upregulation of fatty acid oxidation, succeeded by a short period of fatty acid re-esterification^{7,36,37}. According to Egan and Zierath²⁰, myocellular glycogen replenishment is preferentially activated during the first 24–48 h post-exercise, subsequently causing a temporary increase in fat oxidation during this time, thus substantiating the aforementioned. Withal, normalised concentrations of β -hydroxyhexanoic acid (a β -oxidation intermediate) within the 24 h post-marathon recovery period indicates the restoration of the previously mentioned β -oxidation and redox imbalances^{10,38}, subsequently eliminating the need for alternative fatty acid catabolism pathways, such as α -oxidation, which was previously proposed to be up-regulated during strenuous exercise¹⁰. This hypothesis is further supported by a reduction in α -hydroxyoctanoic acid (an α -oxidation intermediate) and the odd-chain fatty acids (OCFAs; tridecanoic acid, pentadecanoic acid, heptadecanoic acid, and 10-heptadecenoic acid). However, it should be noted that reductions in OCFAs, as well as some of the aforementioned even-chain fatty acids, may also be ascribed to alleviated autophagy³⁹, reduced dietary ingestion of these, and an altered microbial activity⁴⁰.

Ketone production and the TCA cycle. The apparent reduced need for ATP post-marathon³⁵ and the subsequently reduced fuel substrate catabolism during passive recovery is anticipated to cause a reduction in both acetyl-CoA and malonyl-CoA synthesis³⁴. The recovery of the malonic acid concentrations within 24 h post-marathon not only substantiates the latter as it coincides with the previously mentioned transient increase in long-chain fatty acid oxidation, but could also be an indication of said elevated fatty acid synthesis. Furthermore, the restored concentrations of TCA cycle intermediates (α -ketoglutaric acid, fumaric acid, citric acid, and malic acid) and ketones (acetoacetic acid and β -hydroxybutyric acid) to pre-marathon concentrations within

24 h after the marathon (Table 1), support the aforementioned glycaemic flux recovery. This concurs with Nieman et al.⁷, who reported that these pathways remain perturbed for approximately 14 h post-exercise. Additionally, reduced concentrations of ketones, particularly β -hydroxybutyric acid, support the restoration of the previously mentioned redox imbalance^{10,38} and suggest an enhanced metabolic clearance rate⁴¹ in athletes within 24 h post-marathon. β -Hydroxybutyric acid is also a signalling molecule serving as a regulator of gene expression and adaptive responses via the inhibition of class I histone deacetylases, rendering this metabolite invaluable to the recovery process⁴¹.

Amino acid metabolism. In accordance with the carbohydrates and lipids profile, various amino acids (alanine, aspartic acid, glycine, glutamic acid, methionine, phenylalanine, and tyrosine) and associated metabolites (glutaric acid, *p*-hydroxyphenylacetic acid, *p*-hydroxyphenyllactic acid, pyroglutamic acid, and hydroxyproline) recovered to baseline concentrations within 24 h after the completion of the marathon (Table 1), indicating a reduced need for amino acid catabolism during this recovery phase. However, the slight reductions in these amino acids that occur between 24 and 48 h of recovery could be an indication of protein synthesis, as substantiated by the aforementioned uridine/ribose profile (Table 1). Contrarily, slight elevations in recovery time-point hydroxyproline may be indicative of delayed post-marathon collagen catabolism, thus supporting previous literature⁴². Furthermore, the reduced concentrations of α -hydroxybutyric acid (a threonine/methionine metabolism intermediate) within 24 h post-marathon compared to the elevated post-marathon (Day 0) profile correlates with reduced amino acid catabolism and further suggests recovery of previously reported transiently hampered insulin secretion¹⁰, glucose absorption, and imbalance redox potential⁴³.

In addition to the proteinogenic amino acids, elevated concentrations of the branched-chain amino acid (BCAA) valine, in conjunction with reduced BCAA-associated catabolism intermediates (Table 1) such as β -hydroxyisobutyric acid (valine metabolism), α -ethylhydracrylic acid (allo-isoleucine metabolism), and β -hydroxyisovaleric acid (leucine metabolism), are indicative of a reduction in BCAA catabolism. BCAAs are reportedly one of the major factors regulating mammalian target of rapamycin complex 1 (mTOR1), a multi-protein complex responsible for the activation of numerous biological processes imperative to metabolic recovery, including cell proliferation, gene expression for lipid and protein synthesis, as well as the inhibition of cellular autophagy⁴⁴. The proposed activation of these recovery mechanisms is further substantiated by the aforementioned uridine/ribose profile (Table 1), as well as the reduction in various cell membrane-associated intermediates, such as fatty acids (particularly OCFAs), myo-inositol and cholesterol intermediates (squalene and 5-pregnen-3 β ,20 α -diol) observed within 24 h post-marathon. Reduced concentrations of cholesterol intermediates within 24 h after the marathon in comparison to the post-marathon (day 0) concentrations also suggest inactivation of steroidogenesis⁴⁵, which was previously proposed to be induced as a result of endurance activity^{10,37}.

Aminomalonic acid was also observed to recover to pre-marathon concentrations within 24 h post-marathon. Although this metabolite has been associated with oxidative damage to protein/amino acid residues⁴⁶, it has also been isolated from *Escherichia coli*⁴⁷. As such, its precise mechanism of action pertaining to the metabolic recovery process remains to be elucidated.

Conclusion

The results of this investigation suggest that the serum metabolome of athletes largely recovers to baseline (pre-marathon) status within 24 h, with total recovery evident within 48 h post-marathon without the intervention of recovery aids. This can be ascribed to a reduction in ATP requirements during passive recovery, which in turn allows for the activation of anabolic recovery mechanisms or pathways. The possible cascade of events resulting in total metabolome recovery within 24/48 h post-marathon are: (a) reduced energy requirements promoting the restoration of the imbalanced redox potential and intracellular glycaemic flux, thus stimulating glycogenesis. (b) In turn, this further reduces the need for alternative fuel substrate catabolism (of lipids and amino acids) and ketogenesis, (c) activating additional cellular repair mechanisms such as the uridine-dependent nucleotide salvage pathway, mTOR1 stimulation (subsequently inactivating cellular autophagy), as well as possible protein and lipid synthesis/fatty acid re-esterification. Moreover, GM-associated markers emphasize the close metabolic interaction between man and microbe.

Limitations and future prospects

Possible limitations of this investigation may include the inevitable confounder of human genotype/phenotype (sex, age and ethnicity) variation, as well as individual athlete dietary variation. Although the athletes' dietary intake was recorded throughout the investigation, adding dietary restrictions to the already refrained use of recovery aids would be considered too much of an interference to the personalised regimens of these athletes. However, in an attempt to circumvent the variability in participant demographics (age, sex, fitness, running experience, etc.), the study design included repeated measures of the same individuals and the use of statistical methods that consider the dependence between observations (therefore each participant becomes their own control)^{48,49}. Nevertheless, such individual variation allows for a higher level of robustness in the results, which provides a better representation of the true nature of the activated recovery mechanisms as the body attempts to reach homeostasis.

Although the current investigation provided a holistic (untargeted) representation of the metabolome recovery after a marathon, future studies may consider applying more targeted approaches based on these results to possibly identify more personalised/efficient recovery modalities. Additionally, since this investigation was performed on a relatively small cohort, future investigations consisting of larger cohorts and/or serum samples obtained at additional time-points (e.g. 12 h, 36 h, and 72 h post-marathon) are required to validate these findings, ultimately allowing for better metabolic mapping of the in-between stages via the identification of additional

Participant demographical information	Average $\pm$ standard deviation
Age (years)	39 $\pm$ 12
Pre-marathon athlete weight (kg)	72.2 $\pm$ 11.9
Post-marathon athlete weight (kg)	70.8 $\pm$ 11.7
Running experience (years)	8 $\pm$ 7
Marathon experience (races)	21 $\pm$ 40
Finishing time (hh:mm:ss)	04:30:25 $\pm$ 00:36:48

Table 2. Participant demographical information and endurance race experience history.

metabolite markers pertinent to the metabolic recovery trend of endurance athletes. Considering that metabolic differences correlating with gender heterogeneity could not be positively annotated due to limited cohort size, future studies may consider including larger gender-specific cohorts. Lastly, since GM metabolism seems to be an integral part of athletic performance, more targeted analysis of the associated metabolites or using an alternative biological medium (such as faecal matter, urine, etc.) could provide a more perspicuous understanding of the importance of the GM in terms of metabolome recovery.

Methods and materials

Participants and the marathon race. The participants ($n = 16$; 10 males and 6 females) were selected at random from the pool of athletes competing in the Druridge Bay Marathon in Newcastle (UK). The eligibility of the selected participants was assessed by means of a health and dietary questionnaire, after which those individuals with food allergies, cardiovascular complications, musculoskeletal disorders/injuries, or those receiving anti-inflammatory treatment were excluded from the study. In addition, female participants completed a menstruation cycle questionnaire. Considering the ethical implications concerning ethnicity classifications of participants when it is not central to the research topic, as well as the fact that this study was not biased towards any ethnic groups, nor were any of the aims a comparison of ethnicity with respect to the marathon induced changes to the human metabolome, athlete ethnicity was not recorded. All athletes were instructed to abstain from using any recovery modalities/therapies such as cryotherapy, pressure garments, foam rolling, active recovery (moderate exercise bouts), NSAID, vitamins, etc. during the recovery period of this investigation. A summary of participant characteristics is presented in Table 2, while a summary of age, sex, and overall performance distribution of the athletes are presented in Table S2 (Supplementary information). The Druridge marathon entails running four laps around the Country Park (Northumberland coast, UK), which mainly consists of grassy, concrete mixed terrain. Approximately 1.6 km of each lap consists of soft sand (± 6.4 km)⁵⁰ and may contribute to slower finishing times (Table 2).

Sample collection. This investigation forms part of a larger, double-blinded placebo study (Supplementary Fig. S2), in which 31 athletes participating in the 2016 Druridge Bay Marathon either received multiple beetroot ($n = 15$ athletes) or placebo ($n = 16$ athletes) supplements, over a period of 48 h. Blood samples ($n = 124$ samples) were collected 24 h before (as a fasting, yet hydrated, baseline/pre-marathon), immediately after (within 30 min; post-marathon [day 0]), as well as 24 h (day 1 post-marathon) and 48 h (day 2 post-marathon) post-marathon by means of antecubital (basilica vein) venepuncture^{10,50}. Venous sampling was limited to only four sampling intervals considering the impact and inconvenience of increased blood sampling, which would have led to increased participant drop-out. These samples were procured from the Faculty of Health and Life Sciences, Department of Sport, Exercise and Rehabilitation at Northumbria University in Newcastle upon Tyne (UK), and transported to the Laboratory of Infectious and Acquired Diseases, Focus Area: Human Metabolomics at the North-West University in Potchefstroom, as previously described by Stander et al.¹⁰. Considering the aim of the current investigation, only the serum samples ($n = 64$) of the 16 athletes that ingested placebo supplements were included in this study. Furthermore, since this study only focuses on the metabolic adaptations presented during a 48 h recovery period, information regarding additional clinical serum measurements are not included, but are available in a complementary physiological publication based on the same cohort⁵⁰.

Sample extraction and derivatisation. As previously described in detail by Stander et al.¹⁰, all samples along with pooled quality control (QC) samples (pooled 50 μ L of each sample) were subjected to a total metabolome extraction. As part of this process, smaller aliquots of the samples (50 μ L) were prepared, before adding 50 μ L of the internal standard (3-phenylbutyric acid; 0.45 μ g/mL) dissolved in a chloroform:methanol:water (1:3:1) solution. The latter serves as a metabolite conservation step to ensure that larger polar and non-polar compounds and associated metabolites (e.g. amino acids, fatty acids, purines, pyrimidines, alkenes, alkanes etc.) can be analysed using the GCxGC-TOFMS. Protein precipitation of the samples commenced by adding 300 μ L of ice-cold acetonitrile, where after each vial was subjected to a 2 min mixing step (REAX D-91126 vortex; Heidolph Instruments GmbH & Co.KG, Schwabach, Germany) and 10 min centrifugation at 4,000 rpm. The supernatants were extracted, transferred to a clean GC-MS vial, and dried with a light stream of nitrogen gas for 45 min, whilst being housed in a heating block set at 40 $^{\circ}$ C. Once the solvent evaporated, 25 μ L of methoxamine hydrochloride dissolved in pyridine (15 mg/mL) was added to each sample and incubated for 90 min at

50 °C. Lastly, 40 µL of BSTFA enriched with 1% TCMS was added, and samples were left to derivatise/silylate for 60 min at 60 °C. Derivatized samples were then transferred to a clean glass GC–MS vial containing a vial insert.

GCxGC-TOFMS analysis. Samples were injected into the Pegasus 4D GCxGC-TOFMS analytical apparatus (LECO Africa (Pty) Ltd, Johannesburg, South Africa) in a randomised order by using a Gerstel 104 auto-sampler tray (GmbH and co. KG, Mülheim van der Ruhr, Germany), with QC samples preceding approximately every 7th sample for quality assurance purposes. Each sample (1 µL) was injected using a 1:3 split ratio with a constant injector temperature of 270 °C and a carrier gas (purified helium) flow rate of 1 mL/min. The primary oven containing a Restek Rxi-5MS capillary column (30 m; 0.25 µm diameter and 0.25 µm film thickness) was set to operate at an initial temperature of 70 °C before incrementally (4 °C/min) increasing to a final temperature of 300 °C (maintained for 2 min). In addition, the secondary oven, fitted with a Restek Rxi-17 capillary column (1 m; 0.25 µm diameter and 0.25 µm film thickness), was programmed with an initial temperature of 85 °C which was set to incrementally increase with 4.5 °C/min until a final temperature of 300 °C was reached. The thermal modulator, amid the two columns, was set to intermittently pulse streams of hot and cold nitrogen gas for approximately 0.5 s (every 3 s). Mass spectra (ms) of ions (50–800 *m/z* range) was acquired at a rate of 200 ms/s, while the ms of the first 400 s of the run was discarded to exclude extraction solvent elution. However, the latter was not removed from the time axis of the primary column to ensure compound retention time validity. The transfer line and ion source were set to function at 270 °C and 220 °C, respectively, with a detector voltage of 1,600 V and ion filament bias of –70e V. After analytical analysis, the data generated was processed using the ChromaTOF software version 4.7.1 (LECO Corporation), which included ms deconvolution, peak alignment and peak annotation via mass fragment pattern and retention time comparison with in-house libraries compiled from previously injected standards⁵¹. ChromaTof parameter settings included peak widths of 9 and 0.2 for the 1st and 2nd dimension respectively, and a signal-to-noise ratio of 100, with a maximum of 100,000 unknown peaks allowed (to remove bias). All metabolites with molecular masses between 50 and 800 Da were detected with a minimum of 70% similarity match to the in-house libraries for identification. The experimental procedure performed was in accordance with proposed guidelines and regulations, as approved by the North-West University (South Africa) and forms part of the laboratory's standard operating procedures (SOP number: HM-MET-056).

Statistical analysis and group comparison. Prior to data analysis, pooled QC samples were compared to ensure data validity before interpretation. Data analyses commenced with compound normalisation in relation to an internal standard and all identified analytical contaminants/plasticizers/column related metabolites were removed. The data was then subjected to numerous data clean-up steps, including a QC-CV filter (retaining metabolites with CV ≤ 30), QC drift/batch correction (using a pooled QC sample), zero-value replacement (with random, small values along the tail distribution of the data), 50% zero value filter, log transformation and auto-scaling^{48,52–54}. Hereafter the data was subjected to a multi-statistical approach consisting of two main objectives i.e. (1) obtain a global pattern of the metabolic profile of athletes over time and to some extent validate the design against the logical result, as well as (2) determine specific metabolic adaptations between time-points (Fig. 1) to distinguish between sources of variation due to early (day 1 vs day 0) and late (day 2 vs day 0) stages recovery; from full recovery (day 2 vs pre-marathon). As such, statistical analyses consisted of both paired univariate and multivariate approaches, using MATLAB⁵⁵ with a PLS toolbox⁵⁶. In terms of the multivariate analyses, an ML-PCA were used to inspect specific time-point differences in the multivariate space, whereas an ASCA was employed to summarise the overall metabolic perturbation observed over the entire timeframe of the study⁵⁷. Both these methods take the dependence between observations into account. Furthermore, univariate data analyses included a paired *t*-test, corrected for multiple testing by controlling the FDR (limiting to 5%) using the Benjamini–Hochberg procedure⁵⁸, and a dependent effect size test to respectively assess the statistical and practical significance of each metabolite⁴⁸.

Considering the inevitable confounder of inter-individual variation, a repeated measures statistical design was employed to control for the latter as each athlete's metabolic variation is evaluated relative to him/herself. That said, statistical approaches for paired data may not correct for all confounders and as such this design aimed to ensure equal representation across different confounders as indicated in Table S2 (Supplementary information). Although more complex models were considered for variable selection (i.e. repeated measures ANOVA), these models would not be applicable due to the small sample size and were therefore excluded.

Ethical approval. Ethical approval for this investigation, conducted according to the Declaration of Helsinki and International Conference on Harmonization Guidelines, was obtained from the Research Ethics Committee of the Faculty of Health and Life Sciences at the Northumbria University in Newcastle upon Tyne, UK (reference number: HLSTC120716). Informed, written consent was obtained from all individuals included in the study.

Data availability

As the current investigation is part of a larger collaboration study consisting of multiple aims, the dataset of this investigation is not yet publicly available but can be acquired from the corresponding author on reasonable request. The authors declare that all the results included in this study have been presented clearly, honestly and without fabrication, falsification, or inappropriate data manipulation.

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Author contributions

The concept and study design were formulated by D.T.L., Z.S., G.H., T.C., K.M.K., and E.J.S. The samples were acquired from the Northumbria University (G.H., T.C., K.M.K., and E.M.S.) as part of a collaboration agreement with the North-West University. Statistical analyses were performed by M.R. while manuscript drafting, data analysis, and interpretation were performed by Z.S. D.T.L., L.L., and L.J.M. contributed to in-depth data interpretation and the manuscript was repeatedly reviewed by D.T.L. and L.L., of which L.L. provided substantial insights into structural (format) editing. All of the authors reviewed and approved the final draft of this manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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Beetroot juice — a suitable post-marathon metabolic recovery supplement?

Abbreviated title: Beetroot juice aided post-marathon recovery

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ABSTRACT

Background: Red beetroot (*Beta vulgaris L.*) is a dynamic functional food that reportedly exhibits potent anti-inflammatory, antioxidant, vasodilation, and cellular regulatory properties. As such, this vegetable has gained a fair amount of scientific attention as a possible cost-effective supplement to enhance performance and expedite recovery after physical exercise. To date, however, no study has investigated the effects of incremental beetroot juice ingestion on the metabolic recovery of athletes after an endurance race. Considering this, as well as the beneficial glucose and insulin regulatory roles of beetroot, this study investigated the effects of beetroot juice supplementation on the metabolic recovery trend of athletes within 48 h after completing a marathon.

Methods: By employing an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry approach, serum samples (collected pre-, immediately post-, 24 h post-, and 48 h post-marathon) of 31 marathon athletes that ingested a series of either beetroot juice or placebo supplements post-marathon, were analysed and statistically compared.

Results: From the results, it is evident that the metabolic profiles of the beetroot-ingesting cohort recovered to a pre-marathon-related state within 48 h post-marathon, mimicking the metabolic recovery trend observed in the placebo cohort. Nonetheless, since random inter-individual variation was observed immediately post-marathon, only metabolites with large practical significance within 24 h and 48 h post-marathon were considered representative of the effects of beetroot juice on metabolic recovery. These mainly included carbohydrates and odd-chain fatty acids. Although the majority of these were attributed to beetroot content, a great deal of microbial dependence was apparent in these fluctuations.

Conclusion: Regardless, it was concluded, that in the current investigation, beetroot juice did not provide any significant advantages towards expediting metabolic recovery within 48 h post-marathon, when compared to the placebo cohort (unaided recovery).

Keywords: *Beta vulgaris L.*; Endurance running; Recovery; Metabolism; Functional foods.

1. INTRODUCTION

In addition to the well-established physiological (1) and immunological (2) health effects of endurance running races (>5 km), this sport induces a wide variety of metabolic adaptations (3, 4). These adaptations are primarily associated with fuel substrate catabolism and typically include elevated glycolysis, lipolysis, amino acid oxidation, ketogenesis, and tricarboxylate cycle (TCA) intermediates (5-9). Additionally, previously reported elevations in dicarboxylic acids, α -hydroxy acids and odd chain fatty acids (OCFA) coupled with reduced phospholipids, suggest the activation of alternative energy-producing pathways such as ω and/or α -oxidation of fatty acids, as well as autophagy of cellular membranes (5, 10). These adaptations, along with fluctuations in purine and pyrimidine, urea cycle, and reactive oxygen intermediates (5, 6, 8, 9, 11) not only further attest to the extensive energy-taxing nature of endurance running, but also emphasize the metabolic flexibility of athletes to use multiple fuel substrates during endurance races, to supply the necessary energy to complete the event. Based on previous literature, these metabolic adaptations recover to pre-exercise-related levels within 3–72 h after cessation of endurance exercise (12-15), depending on the intensity, duration, and distance of the running event. Post-exercise metabolic restoration is mainly attributed to a reduction in energy requirements, which leads to the inactivation of catabolic pathways required for energy production, and the activation of anabolic pathways required for recovery, including the nucleotide salvage pathways, glycogenesis, lipogenesis and protein synthesis (12, 13, 15).

Besides the potentially detrimental, performance diminishing effects induced, endurance races are still considered a popular leisure activity, globally. As such, a growing body of research is aimed at identifying more cost-effective strategies that may expedite the recovery process, and subsequently enhancing athlete performance. One of these strategies includes pre- and/or post-exercise ingestion of supplements that either consists of or contains functional foods. Functional foods are primarily defined as any natural and/or fortified foods, rich in bioactive compounds that possess an added advantage to human health besides its nutritional value (16, 17). Bioactive phytonutrients (i.e. polyphenols, carotenoids, curcuminoids, anthocyanins, vitamins, etc.) found in fruits and vegetables, reportedly enhance and maintain cardiovascular health, reduce cholesterol, while also preventing DNA damage,

diabetes and certain cancers (18-20). Besides these beneficial health attributes, phytonutrient-rich fruits and vegetables are also well-known for their radical scavenging, antioxidant, anti-inflammatory and anti-viral/microbial properties (21) that may aid in performance-enhancing and/or physiological recovery of athletes during and/or after exercise. To date, some of the foods that have been investigated for this particular purpose include cherries, blueberries, carrots, bananas, beetroot, blackcurrants, pomegranates, pears, etc. (22-28). Moreover, it has been proposed that the anti-oxidant and anti-inflammatory capacity of beetroot (*Beta vulgaris L.*) surpasses that of most other fruits and vegetables due to its abundant betalain, among other phytochemicals (polyphenols, vitamin C, rutin, epicatechin, etc.) content, which inhibits cyclooxygenase activity and disrupts nuclear factor kappa-light-chain-enhancer of activated B cells (20). In addition to these pigment imposing betalains, beetroot is also considered a Class A performance-enhancing supplement (supported by adequate scientific research) by the Australian Institute of Sport (29) due to its nitrate donating abilities. Nitrate reportedly enhances nitric oxide bioavailability via the nitrate-nitrite-nitric-oxide pathway, subsequently enhancing vasodilation, cellular respiration regulation and neurotransmission (22, 30, 31). Besides the successful application of beetroot juice as a performance-enhancing supplement, controversial evidence exists regarding its ability to expedite the physiological recovery process after exercise (22, 32). To our knowledge, no study has investigated the capacity of beetroot juice to facilitate metabolic recovery after endurance running.

Thus, in this double-blinded, placebo-controlled investigation, an untargeted two-dimensional gas chromatography time-of-flight mass spectrometry approach (GCxGC-TOFMS) metabolomics approach was used to determine whether or not beetroot juice ingestion possesses an added advantage towards metabolic recovery when compared to unaided recovery within 48 h post-marathon. This knowledge could not only further improve the current understanding of beetroot aided recovery, but also provide clues to new, more effective dose-dependent supplementation beetroot ingestion after exercise.

2. METHODS AND MATERIALS

Considering that this project forms part of a larger study that consists of multiple (inter-disciplinary)

aims, additional clinical and physiological measurements (22), as well as complementary metabolic investigations (5, 15) based on sub-divisions of the current cohort, have already been published and may be referred to for additional information not pertinent to this investigation.

2.1. Participants

The participants included in this investigation were selected at random and participation was completely voluntary. Prior to the marathon, all participants were required to complete a health and dietary questionnaire (with an additional menstrual cycle questionnaire for female participants), of which individuals receiving or using any anti-inflammatory treatments, chronic medication, as well as those with any food allergies, cardiovascular complications or musculoskeletal disorders and injuries were excluded from the study. Furthermore, all athletes were instructed to refrain from exercising and/or using any alternative recovery modalities (heat, cryotherapy, inflammatory drugs, antioxidant vitamins, compression garments, etc.) during the recovery period of this investigation. Withal, the use of anti-bacterial mouth wash was prohibited as a means of conserving the proposed bacterial nitrate-nitrite conversion of beetroot juice in the oral cavity. All the participants gave written and informed consent before the commencement of any analysis. An overview of the participant characteristics/demographics is presented in Table 1.

Table 1: A summary of the participant characteristics of the placebo and beetroot ingesting cohorts.

Participant demographical information	Placebo cohort (n=16)	Beetroot cohort (n=15)
	Average $\pm$ standard deviation	
Age (years)	39 $\pm$ 12	42 $\pm$ 10
Pre-marathon athlete weight (kg)	72.2 $\pm$ 11.9	70.3 $\pm$ 7.9
Post-marathon athlete weight (kg)	70.8 $\pm$ 11.7	69 $\pm$ 7.4
Marathon experience (years)	8 $\pm$ 7	11 $\pm$ 10
Marathon experience (races)	21 $\pm$ 40	10 $\pm$ 12
Finishing time (hh:mm:ss)	04:30:25 $\pm$ 00:36:48	04:07:08 $\pm$ 00:39:16

2.2. Clinical samples and supplementation

Blood samples of 31 athletes (19 males;12 females) were obtained (antecubital venesection) 24 h before

(P0) and immediately after (P1), as well as 24 h (P2) and 48 h (P3) after completing the Druridge Bay Marathon (Northumberland, UK) (22). During the two consecutive days following the race, athletes received either beetroot juice (n=15 athletes; 9 males and 6 females) or a placebo supplement (n=16 athletes; 10 males and 6 females). These supplements were indistinguishable in appearance and were consumed as follows: 3x 250 ml supplements on the day of the marathon (immediately after P1 sampling, ± 3 h post-race, and at 20:00), 3x 250 ml supplements on the first day after the marathon (upon waking-up, with lunch, and with supper), and 1x 250 ml supplement upon waking on the second day post-marathon. A detailed summary of the nutrient composition of the supplements is provided by Clifford, Allerton (22). P0 samples (hydrated yet fasting [for at least 4 h] state) were collected on the day preceding the race, whereas P1 samples were acquired within 30 min after completing the race, thus dictating the approximate time of P2 and P3 collection. All the blood samples were collected in 10 mL vacutainer vials and placed on ice before being transported to the Northumbria University (Newcastle upon Tyne, UK), Faculty of Health and Life Sciences (Department of Sport, Exercise and Rehabilitation) laboratory, for further processing. Initial sample processing included clotting at room temperature for 30 min, followed by a 10 min centrifugation step (3 000 g). The serum (supernatant) was then extracted, immediately frozen (-80°C), and transported on dry ice to the North-West University, Human Metabolomics: Laboratory of Infectious and Acquired Diseases for metabolomics analyses. All samples were stored at -80°C until analysis commenced.

2.3. Total metabolome extraction and derivatisation

As previously described (5, 15), all samples, including pooled quality control samples (containing 50 μL of each sample), were subjected to an in-house total metabolome extraction (SOP number: HM-MET-056) and traditional TMCS derivatisation before being analysed. To summarise; 50 μL of internal standard (3-phenylbutyrate; 0.45 $\mu\text{g}/\text{ml}$), dissolved in a chloroform:methanol:milliQ water (1:3:1) solution, was added to smaller aliquots (50 μL) of the samples. While on ice, 300 μL of ice-cold acetonitrile was added to the aliquots, whereupon it was mixed for 2 min (REAX D-91126 vortex; Heidolph Instruments GmbH & Co.KG, Schwabach, Germany), and centrifuged for 10 min at 4000 rpm. The supernatants of the samples were then extracted, transferred to glass GC-MS vials,

placed in a heating block set to 40°C, and dried under a stream of nitrogen gas for approximately 45 min. Using a Hamilton syringe, 25 µl of methoxamine hydrochloride (dissolved in 15 mg/ml pyridine) was added to each vial, which proceeded to incubate for 90 min at 50°C. Finally, samples were derivatised with 40 µl BSTFA (enriched with 1% TMCS) for 60 min at 60°C, before being transferred a new GC vial containing a vial insert.

2.4. GCxGC-TOFMS analysis and processing

The randomised samples were injected (1 µl; 1:3 split ratio) into the Pegasus 4D GCxGC-TOFMS system (LECO Africa (Pty) Ltd, Johannesburg, South Africa), using the Gerstel auto-sampler (Gerstel GmbH and co. KG, Mülheim van der Ruhr, Germany). The carrier gas (purified helium) was set to flow at a constant rate of 1 ml/min, while the injector temperature was held at 270°C. The primary oven, containing a Restek Rxi-5MS capillary column (30 m; 0.25 µm diameter and 0.25 µm film thickness), was programmed with an initial temperature of 70°C, which incrementally (4°C/min) increased until a final temperature of 300°C was reached (maintained for 2 min). The secondary oven, containing a Restek Rxi-17 capillary column (1 m; 0.25 µm diameter and 0.25 µm film thickness), was set at 85°C, which increased with 4.5°C/min until a final temperature of 300°C was reached (maintained for 2 min), while the thermal modulator pulsed cold and hot streams nitrogen gas every 3 s for a duration of 0.5 s. The mass spectra (ms) of the first 400 s of each run was discarded (regarded as solvent delay), whereafter ms of ions (50–800 m/z) were acquired at 200 ms/s. Furthermore, the transfer line and ion source were held at 270°C and 220°C, respectively, with a detector voltage of 1600 V and filament bias of -70 eV. The data generated from the GCxGC-TOFMS was processed (deconvolution, peak alignment and identification) using the ChromaTOF Software (LECO Corporation), as described by Stander, Luies (5).

2.5. Data processing and statistical analyses

The dataset obtained was normalised relative to the internal standard, and plasticizers, analytical contaminants, and column-related compounds were removed. Hereafter, a 50% zero value filter, zero value replacement (with random values below the detection limit), 50% quality control coefficient of

variation (QC-CV) filter (retaining metabolites with a $CV \leq 50\%$), log transformation, and auto-scaling were performed.

Following these clean-up steps, the data was subjected to a variety of multivariate and univariate statistical methods using MATLAB (33) (in conjunction with a PLS (34) toolbox), as a means of selecting those metabolites pertinent to the aim of this investigation. In order to comprehensively address the aim of this investigation, multiple statistical objectives, and therefore comparisons, were required. In summary, paired statistical analysis of the beetroot juice-ingesting cohort was performed to confirm whether this cohort indeed recovered to a pre-marathon-related state within 48 h (statistical objective A), as has already been confirmed for the placebo group (15). Hence, the P2 and P3 serum metabolite profiles of the beetroot-ingesting cohort were respectively compared to that of the P0 profile to identify any differentiating metabolites which would oppose metabolic recovery. Multivariate analyses included multilevel (ML) principal component analyses (PCA) and ML-partial squares discriminant analysis (ML-PLS-DA), while univariate analyses consisted of a paired t-test, and effect size tests, to assess statistical and practical significance respectively. To control for false discovery rates (FDR) associated with large scale multiple testing, as in the case of untargeted metabolomics datasets, t-test p -values were adjusted using the Benjamini-Hochberg procedure (limiting FDRs to 5%). All metabolites (P0 vs P2 and P0 vs P3) with a BH adjusted p -value ≤ 0.05 and an effect size d -value ≥ 0.5 were deemed significant.

Furthermore, in order to determine whether or not beetroot juice ingestion expedites the metabolic recovery trend of athletes within 48 h post-marathon, unpaired statistical analyses of the beetroot cohort vs placebo cohort were performed (statistical objective B). Foremost, the metabolic progression for both treatment groups over time was compared by using statistical models that accounted for the entire experimental design and dependencies between measures, i.e. a two-way repeated-measures analysis of variance (RM ANOVA), and an unfolded PCA. The latter transforms a three-dimensional tensor into a two-dimensional matrix (Fig. S1), thus allowing for PCA (35). To supplement these comprehensive statistical methods, recovery time-point-specific inter-cohort comparisons were performed to assess day-specific variation that may be apparent between the cohorts. For this, multivariate statistical

methods included PCA, PLS-DA, whilst univariate methods comprised an independent samples t-test (FDR's controlled to 5% according to the BH-procedure), and independent effect size tests based on Cohen's d -values. Here, variables were selected based on a BH adjusted p -value ≤ 0.05 or a Cohen's d -value ≥ 1.0 , thus considering both statistical and practical significance. However, as opposed to the equal statistical and practical relevance of marker selection in objective A, this objective's selection was more stringent on practical relevance to capture slight differences that may be of practical importance.

Since the cohort sizes of this investigation are relatively small, multivariate models are less readily validated and could therefore only be used to visualise trends and variation, whilst univariate models, better equipped to avoid false discoveries, were employed for variable selection in both statistical objectives.

3. RESULTS

When comparing both the P2 and P3 serum metabolite profiles of the beetroot cohort to the corresponding P0 profiles (statistical objective A), no metabolite markers were considered statistically significant (BH p -value ≤ 0.05 and d -value ≥ 0.5), suggesting that the metabolome of the beetroot cohort recovered to a baseline-related state within 48 h post-marathon. This was further indicated by the confined positioning of the respective ML-PCA of each recovery time-point relative to the P0 ellipsoids, as presented in Fig. 1.

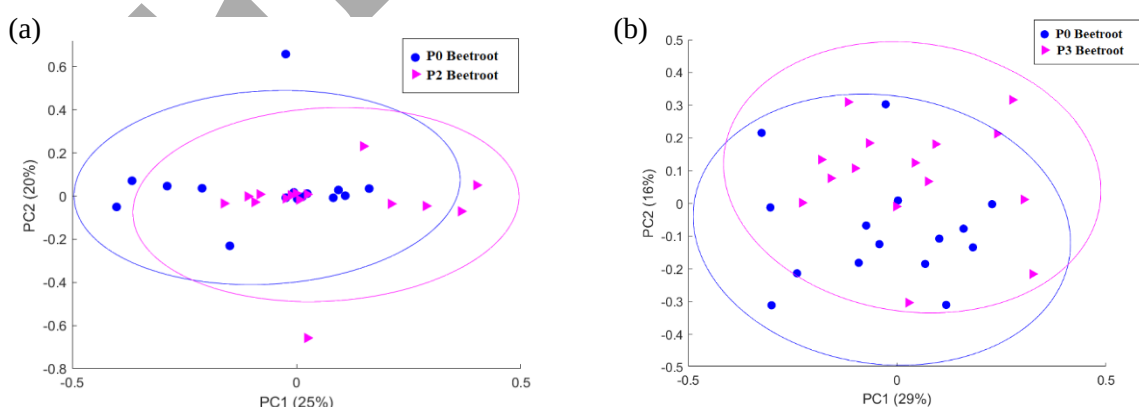


Fig. 1: Multi-level principal component analysis plot of the pre-marathon [P0] serum metabolite profiles of the beetroot group relative to the (a) 24h post-marathon [P1] and (b) 48h post-marathon metabolite profiles [P2], profiles of the same group. Abbreviations: PC: principal component.

Since these results correspond with the findings of Stander, Luies (15) that indicated overall metabolic restoration of the placebo athletes within 48 h post-marathon, an unfolded PCA was constructed to visualise the time-dependent global metabolic recovery trend of the two cohorts relative to each other (Fig. 2) (statistical objective B).

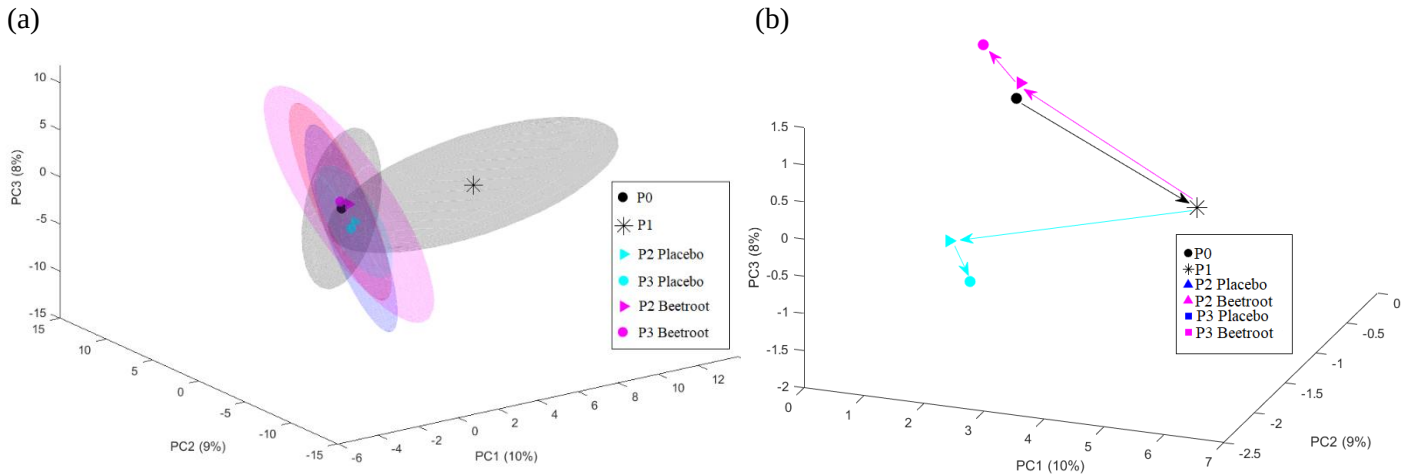


Fig. 2: Unfolded principal component analysis (a) with, and (b) without confidence intervals, depicting the global recovery trend of the beetroot and placebo cohort over time. Abbreviations: P0: pre-marathon; P1: post-marathon; P2: 24 h post-marathon; P3: 48 h post-marathon; PC: principal component.

From this, it is evident that the most significant effect emanated from the marathon perturbation (5), after which both groups returned to a pre-marathon-related state within ± 24 h (15). Intensely overlapped confidence intervals of the recovery time-points of the beetroot juice and placebo cohorts suggest little to no variation between groups at these times (Fig. 2a). However, upon removal of confidence intervals, P2 and P3 centroids of the beetroot cohort are seemingly slightly closer to that of P0 (Fig. 2b), which could be indicative of small inter-cohort differences. As such, a two-way RM ANOVA was employed, and 16 metabolites were identified as having a statistically significant (p -value ≤ 0.05) interaction (between time and intervention) and/or intervention effect (Table 2). Considering that this univariate method focuses on relatively small differences within the entire study design (P0–P3), the RM ANOVA alone does not discriminate the precise origin of the metabolic fluctuation observed. Consequently, pairwise comparisons of the different time-points were employed to determine day-specific recovery effects. Whilst the PCA plots of each time-point comparison (Fig. S2) did not visually portray significant differences between the groups at the different time-points, Cohen’s d -

values for the majority of these RM ANOVA metabolites indicated random, moderate-to-large practical variance amongst the groups (Table 2). A more detailed table including RM ANOVA *p*-values as well as average metabolite concentrations and standard deviations can be found in Table S1.

Table 2: Metabolites varying significantly between the beetroot and placebo cohorts, according to RM ANOVA and day-specific pairwise comparisons.

Metabolite (PubChem ID)	P0 Placebo vs P0 Beetroot juice		P1 Placebo vs P1 Beetroot juice		P2 Placebo vs P2 Beetroot juice		P3 Placebo vs P3 Beetroot juice	
	BH p-value	Cohen's d-value	BH p-value	Cohen's d-value	BH p-value	Cohen's d-value	BH p-value	Cohen's d-value
α -Oleoylglycerol (5319879) ^b	0.89	0.65	0.92	0.31	1.00	0.13	0.89	0.19
β -Hydroxydecenedioate (121232666) ^b	0.92	0.33	0.97	0.05	1.00	0.37	0.56	0.44
β -Hydroxyphenylacetate (12122) ^a	0.97	0.22	0.92	0.60	1.00	0.34	0.27	0.85
β -Hydroxyvalerate (107802) ^a	0.92	0.33	0.92	0.54	1.00	0.58	0.31	0.64
<i>p</i> -Hydroxyphenylacetate (127) ^a	0.89	0.61	0.92	0.31	1.00	0.65	0.30	0.70
Arabitol (43925) ^{a,b,*}	0.89	0.45	0.96	0.12	0.03	1.40	2.1x10 ⁻³	1.73
Eicosanoate (10467) ^a	0.89	0.58	0.92	0.46	1.00	0.36	0.50	0.56
Glycerol (753) ^b	0.97	0.13	0.92	0.57	1.00	0.24	0.99	0.02
Hippurate (464) ^a	0.89	0.59	0.92	0.39	1.00	0.19	0.27	0.86
Mannitol (6251) ^a	0.99	0.11	0.92	0.37	1.00	0.55	0.37	0.59
Nonanoate (8158) ^{a,*}	0.89	0.71	0.92	0.84	0.57	1.02	2.6x10 ⁻³	1.63
Oxalate (971) ^a	0.89	0.40	0.92	0.79	1.00	0.49	0.92	0.12
Rhamnose (25310)	0.89	0.63	0.92	0.46	1.00	0.65	0.88	0.22
Threonate (21145021) ^{a,b}	0.88	0.61	0.89	1.03	1.00	0.30	0.52	0.58
Undecanoate (8180) ^{a,*}	0.40	1.24	0.92	1.07	1.00	0.35	0.04	1.38
Xylose (135191) ^{a,b,*}	0.89	0.56	0.92	0.29	0.21	1.04	0.53	0.46

Footnote: Intervention (^a), interaction (^b) effects, and day-specific significance (*) are indicated in the first column, while day-specific *p*- and *d*-value significance is indicated with grey shading.

In light of the pre-existing individual variance (particularly at P1), as well as the lack in statistical significance of metabolites (BH *p*-value ≤ 0.05) during the recovery period (P2 and P3), only those

metabolites with a large effect (d -value ≥ 1.0) at the respective recovery time-points were considered relevant in terms of understanding any possible treatment-induced differences at various stages of recovery (P2 and P3) when comparing the beetroot and the placebo cohort. Based on this, only four metabolites (arabitol, nonanoate, undecanoate and xylose) were identified as significantly altered when comparing these cohorts (indicated with an asterisk in Table 2), while significant p - and d -values are emphasized with grey shading.

4. DISCUSSION

Beetroot juice has gained a considerable amount of attention as a possible performance optimisation and recovery enhancing sports supplement due to its potent reactive oxygen scavenging properties and its ability to promote vasodilation, regulate cellular respiration and reduce inflammation (20, 36). However, limited literature is available pertaining to the metabolic recovery effects of beetroot juice after endurance running exercises. In order to completely ascertain whether or not beetroot juice possess an added advantage in terms of metabolic recovery within 48 h post-marathon, it is imperative to also comprehend the unaided metabolic recovery of athletes within the same timeframe. These results have been published as part of a previous investigation (15), and will therefore only be summarised here. Unaided metabolic recovery was attained within 24 h post-marathon (with exception of xylose), and included reductions in carbohydrates, fatty acids and TCA cycle intermediates, along with elevations in amino acids. Recovery of these was ascribed to a reduction in energy requirements, leading to the activation of glucogenesis, fatty acid re-esterification, protein synthesis, cellular membrane restoration and the possible activation of nucleotide salvage pathways (15, 37). In this investigation, a comprehensive comparison of the recovery trends of the beetroot juice and placebo cohorts did not vary significantly (Fig 2), thus indicating no added advantage of beetroot juice ingestion in terms of metabolic recovery after the marathon. However, when employing a two-way RM ANOVA, significant (albeit small) concentration fluctuations were observed in 16 metabolites (Table 2). Whilst these fluctuations were significant when considering the entire study design, pairwise analysis of the respective time-points later revealed that a large portion is owed to P1 (and to some extent P0) variation between athletes. Besides the inevitable confounder of genetic, age and gender variation, these P1

disparities are to be expected since the dietary regimens of athletes were not restricted and/or controlled at either of these time-points as a means of providing a more relatable, robust representation of the marathon perturbation (5). Regardless, while the substantial impact of the marathon perturbation totally overshadowed these differences (5), the effect of beetroot juice supplementation is evidently negligible, thus supporting previous literature (22, 38). As such, it is unsurprising that only four metabolite markers were identified to vary significantly at the different stages of recovery (P2 and P3). These metabolites (arabitol, xylose, nonanoate, and undecanoate) will be comprehensively discussed below.

In addition to the initial increase of arabitol in both cohorts during the marathon (5), this metabolite continued to increase in the beetroot-ingesting cohort during the recovery period, while contrarily decreasing in the placebo cohort (15). Although at first glance this may be associated with low-calorie sweetener ingestion, as previously reported to occur during the marathon (5), *Love Beets* product labels indicated that no additional sweeteners were added to this 99% organic beetroot juice supplement (with 1% lemon juice as a stabiliser). However, considering that red beetroot pectin fibres are rich in various sugars including xylose, rhamnose, mannose and especially, arabinose (39), it can be deduced that although not directly added to the supplement, arabitol may have been produced via the reduction of arabinose in the polyol pathway present in humans and various intestinal yeasts (40, 41). Furthermore, it has been reported that intestinal bacteria preferentially ferment arabinose over other sugars such as xylose (42), thus further ascribing the elevated xylose bioavailability at P2 in this cohort. In addition, xylose can serve as a minor precursor for ribose, the latter which is a key component in the uridine-dependent salvage pathway during recovery (15). As such, since beetroot phytonutrients, including betaines (43), folate (44), and glutamine (39), can assist in the process of nucleotide restoration, the observed P2 elevations in xylose could also partially be indicative of the role of beetroot-promoting nucleotide synthesis via alternative constituents, subsequently reducing the incorporation of xylose to produce ribose. Nevertheless, whilst still being significantly elevated compared to the placebo cohort, slight P3 reductions (compared to P2) in both arabitol (significant) and xylose (non-significant) were observed in the beetroot juice cohort and may be attributed to the fact that only a single beetroot supplement (upon waking) was ingested on the final day of the investigation (P3), as opposed to the

three servings at P1 and P2. Nevertheless, arabitol has been associated with numerous health benefits, of which the prevention of blood sugar dysregulation and fat deposition in the intestinal tract (41) is the most applicable to post-race metabolic recovery.

Furthermore, opposing fluctuations in OCFA (nonanoate and undecanoate) concentrations were observed during recovery. In general, reductions in undecanoate (C₁₁) observed in both cohorts within 24 h post-marathon, is most likely due to residual catabolism of the accumulated fatty acids via α -oxidation (5). This is further supported by elevations in nonanoate (C₉) in both cohorts during this time since it is a downstream product of undecanoate catabolism. Nevertheless, mitigation of OCFA catabolism is evident by the reduction in both nonanoate and undecanoate concentrations in the placebo cohort within 48 h post-marathon. Contrarily, however, both of these OCFAs are elevated in the beetroot cohort 48 h post-marathon, possibly indicating the activation of OCFA synthesis in this cohort. The proposed mechanism of action for this includes the fermentation of beetroot sugar-content to propanoate via intestinal bacteria (*Bacterioidetes*, *Clostridium*, *Lactobacillus*, etc.) (45), which can be further utilised for OCFA synthesis. Argumentatively, placebo supplements evidently contained the same total amount of sugar, however, this did not correlate an increase in OCFAs 48 h post-marathon. This may not only be ascribed to the increased propionate-producing bacterial activity (i.e. *Lactobacillus*) induced by beetroot pectin ingestion (45), but may also arise as a result of the polyphenol content in beetroot which promotes the ability to reduce effective carbohydrate absorption in the intestines (46), subsequently prolonging the exposure of beetroot sugars to gut microbes for fermentation. Besides the possible contribution of OCFA to phospholipid content (i.e. cellular membrane restoration), recent findings have observed an inversed correlation of OCFA and leptin as well as plasminogen-inhibitor-1 (47), further suggesting its role in energy homeostasis regulation and the activation of fibrinolysis. Nevertheless, whether or not elevated OCFAs has an additional advantage pertaining to athlete recovery, remains to be determined.

5. CONCLUSION

This investigation not only supported previous findings of total metabolic recovery of athletes within

48 h post-marathon, but further indicated that beetroot ingestion does not expedite the metabolic recovery process. Although several significant, albeit small, metabolic fluctuations between cohorts were selected based on univariate statistics, the majority revealed considerable random variation at P1, which is owed to opposing athletic regimens and inherent biological and physiological aspects. As such, only metabolites with large practical significance at the respective days of the supplement interventions (P2 and P3) were considered when addressing the aim of this investigation. Of the four metabolites identified, the majority were associated with the glycaemic content of red beetroot, as well as the increased microbial activity stimulated by this vegetable. Whether these may provide an additional advantage during an extended period of recovery remains unknown, but as of immediate recovery, no metabolic advantage linked to these were annotated. However, the lack of beetroot juice effectiveness may stem from dose-dependent absorption of phytonutrients central to the recovery process, and the considerable dependence on the composition and fermentation preferences of gut-microbes.

6. LIMITATIONS AND FUTURE PROSPECTS

As with all human-based investigations, an inevitable limitation includes human genotype/phenotype (sex, age, and ethnicity [latter not recorded]) variation confounders, as well as possible dietary variation among athletes. Considering the list of restrictions in terms of recovery modalities and prohibition of anti-bacterial mouth wash provided to the athletes, additional dietary restrictions would have potentially imposed on personalised regimens of these athletes, leading to a reduction in the already confined cohort size. Nevertheless, although not controlled, dietary intake was recorded for the entire duration of this investigation in an attempt to circumvent this. However, these individual variations may introduce a higher level of robustness into the results, providing a more realistic and relatable representation of the metabolic effects of incremental beetroot juice supplementation after a marathon. Since the cohort size of this investigation is considered relatively small in terms of human-based studies and the intricate accompanying statistical approaches needed for analysing the data, future studies consisting of a larger cohort (particularly per gender), could further validate these findings and possibly determine the metabolic effects of beetroot juice in a gender-dependent manner. Additionally, the lack of efficacy of

beetroot juice observed in this study may be due to the rapid restoration of the metabolism even when not subjected to aiding strategies, therefore future studies may consider observing the effect(s) of beetroot supplements at shorter incremental time-points along this recover process (i.e. 6 h, 12 h, 18 h, etc.). Furthermore, since the microbiome of athletes seems to be a key component in the conversion of red beetroot nitrate, the fermentation of sugar components, and metabolism of polyphenols, metabolic studies investigating the effects of beetroot on the gut microbiome and microbial function, could determine the fermentation preferences and possible causes for metabolic delays pertinent to phytonutrient uptake.

7. ABBREVIATIONS

BH: Benjamini-Hochberg; DNA: Deoxyribonucleic acid; FDR: False discovery rates; GC: Gas chromatography; GCxGC-TOFMS: Two-dimensional gas chromatography time-of-flight mass spectrometry; ML: Multilevel; OCFA: Odd-chain fatty acids; PCA: Principal component analysis; PLS-DA: Partial least squares discriminant analysis; RM ANOVA: Repeated-measures analysis of variance; TCA: Tricarboxylic acid.

8. ADDITIONAL INFORMATION

8.1. Author contribution

The concept and study design were formulated by DTL, ZS, GH, TC, KMK, and EJS. The samples were acquired from the Northumbria University (GH, TC, KMK, and EMS) as part of a collaboration agreement with the North-West University. Statistical analyses were performed by MR while manuscript drafting, data analysis, and interpretation were performed by ZS. DTL and LL contributed to in-depth data interpretation and the manuscript was repeatedly reviewed by DTL and LL, of which LL provided substantial insights into structural (format) editing. All of the authors reviewed and approved the final draft of this manuscript.

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8.4. Data availability statement

As the current investigation is part of a larger collaboration study consisting of multiple aims, the dataset of this investigation is not yet publicly available but can be acquired from the corresponding author on reasonable request. The authors declare that all the results included in this study have been presented clearly, honestly and without fabrication, falsification, or inappropriate data manipulation.

9. COMPLIANCE WITH ETHICAL STANDARDS

9.1. Conflict of interest

The authors declare that there are no conflicts of interest and that this manuscript has not been submitted/published elsewhere.

9.2. Ethical approval

Ethical approval for this investigation, conducted according to the Declaration of Helsinki and International Conference on Harmonization Guidelines, was obtained from the Research Ethics Committee of the Faculty of Health and Life Sciences at the Northumbria University in Newcastle upon Tyne, UK (reference number: HLSTC120716). Informed, written consent was obtained from all individuals included in the study.

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11. SUPPLEMENTARY MATERIAL

Table S1: Average concentrations and RM ANOVA p-values the metabolites selected to be significant pertaining to the aim of this investigation

Metabolite (PubChem ID)	Group	RM ANOVA		P0	P1	P2	P3
		Intervention p-value	Interaction p-value	Average concentration $\pm$ standard deviation			
α -Oleoylglycerol (5319879)	Placebo	0.42	0.01	$0.001 \pm 3.8 \times 10^{-4}$	$0.001 \pm 3.6 \times 10^{-4}$	$4.3 \times 10^{-4} \pm 3.1 \times 10^{-4}$	$0.001 \pm 3.9 \times 10^{-4}$
	Beetroot			0.001 ± 0.001	$4.1 \times 10^{-4} \pm 2.6 \times 10^{-4}$	$4.8 \times 10^{-4} \pm 3.2 \times 10^{-4}$	$0.001 \pm 3.9 \times 10^{-4}$
β -Hydroxydecenedioate (121232666)	Placebo	0.58	0.04	0.005 ± 0.001	0.006 ± 0.002	0.007 ± 0.002	0.005 ± 0.001
	Beetroot			0.006 ± 0.002	0.006 ± 0.002	0.006 ± 0.001	0.006 ± 0.003
β -Hydroxyphenylacetate (12122) ^a	Placebo	0.01	0.07	$3.1 \times 10^{-5} \pm 4.6 \times 10^{-5}$	$2.8 \times 10^{-5} \pm 4.1 \times 10^{-5}$	$1.6 \times 10^{-5} \pm 3.1 \times 10^{-5}$	$2.0 \times 10^{-5} \pm 3.6 \times 10^{-5}$
	Beetroot			$4.3 \times 10^{-5} \pm 6.4 \times 10^{-5}$	$1.1 \times 10^{-4} \pm 1.3 \times 10^{-4}$	$3.2 \times 10^{-5} \pm 4.3 \times 10^{-5}$	$5.9 \times 10^{-5} \pm 4.3 \times 10^{-5}$
β -Hydroxyvalerate (107802)	Placebo	0.02	0.72	$5.3 \times 10^{-4} \pm 4.2 \times 10^{-4}$	$6.0 \times 10^{-4} \pm 3.9 \times 10^{-4}$	$6.8 \times 10^{-4} \pm 4.5 \times 10^{-4}$	$5.2 \times 10^{-4} \pm 1.7 \times 10^{-4}$
	Beetroot			0.001 ± 0.001	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001
<i>p</i> -Hydroxyphenylacetate (127)	Placebo	0.02	0.92	0.001 ± 0.001	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001
	Beetroot			0.002 ± 0.001	0.003 ± 0.001	0.002 ± 0.001	0.002 ± 0.001
Arabitol (43925)	Placebo	2.9×10^{-4}	1.4×10^{-8}	0.004 ± 0.001	0.005 ± 0.002	0.004 ± 0.001	0.003 ± 0.001
	Beetroot			0.005 ± 0.002	0.005 ± 0.002	0.007 ± 0.002	0.007 ± 0.002
Eicosanoate (10467)	Placebo	0.01	0.95	$4.2 \times 10^{-4} \pm 2.3 \times 10^{-4}$	$0.001 \pm 4.1 \times 10^{-4}$	$4.6 \times 10^{-4} \pm 2.9 \times 10^{-4}$	$3.7 \times 10^{-4} \pm 2.0 \times 10^{-4}$
	Beetroot			$0.001 \pm 4.4 \times 10^{-4}$	$0.001 \pm 3.6 \times 10^{-4}$	0.001 ± 0.001	$0.001 \pm 2.8 \times 10^{-4}$
Glycerol (753)	Placebo	0.33	0.03	0.084 ± 0.031	0.492 ± 0.157	0.083 ± 0.032	0.092 ± 0.036
	Beetroot			0.092 ± 0.052	0.399 ± 0.114	0.091 ± 0.032	0.094 ± 0.047
Hippurate (464)	Placebo	0.02	0.73	0.002 ± 0.002	0.003 ± 0.004	0.003 ± 0.004	0.001 ± 0.002
	Beetroot			0.003 ± 0.003	0.005 ± 0.006	0.004 ± 0.004	0.004 ± 0.003
Mannitol (6251)	Placebo	0.01	0.06	0.003 ± 0.004	0.005 ± 0.004	0.004 ± 0.003	0.003 ± 0.003
	Beetroot			0.004 ± 0.004	0.01 ± 0.013	0.02 ± 0.03	0.008 ± 0.009
Nonanoate (8158)	Placebo	1.7×10^{-3}	0.13	0.004 ± 0.002	0.004 ± 0.002	0.004 ± 0.001	0.003 ± 0.001
	Beetroot			0.005 ± 0.002	0.005 ± 0.001	0.005 ± 0.001	0.005 ± 0.001
Oxalate (971)	Placebo	0.04	0.24	0.013 ± 0.005	0.015 ± 0.006	0.014 ± 0.004	0.016 ± 0.007
	Beetroot			0.016 ± 0.006	0.02 ± 0.006	0.016 ± 0.005	0.015 ± 0.004
Rhamnose (25310)	Placebo	0.01	0.57	0.001 ± 0.001	0.002 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
	Beetroot			0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.001
Threonate (21145021)	Placebo	0.07	0.02	0.01 ± 0.004	0.014 ± 0.005	0.011 ± 0.004	0.011 ± 0.005
	Beetroot			0.013 ± 0.005	0.018 ± 0.005	0.011 ± 0.002	0.013 ± 0.003
Undecanoate (8180)	Placebo	7.9×10^{-4}	0.15	$7.7 \times 10^{-5} \pm 5.7 \times 10^{-5}$	$1.3 \times 10^{-4} \pm 4.7 \times 10^{-5}$	$1.2 \times 10^{-4} \pm 6.2 \times 10^{-5}$	$9.1 \times 10^{-5} \pm 3.6 \times 10^{-5}$
	Beetroot			$1.5 \times 10^{-4} \pm 4.8 \times 10^{-5}$	$1.8 \times 10^{-4} \pm 3.9 \times 10^{-5}$	$1.4 \times 10^{-4} \pm 5.5 \times 10^{-5}$	$1.4 \times 10^{-4} \pm 3. \times 10^{-5}$
Xylose (135191)	Placebo	0.01	0.02	0.016 ± 0.008	0.014 ± 0.008	0.007 ± 0.004	0.011 ± 0.007
	Beetroot			0.023 ± 0.013	0.012 ± 0.006	0.02 ± 0.012	0.017 ± 0.015

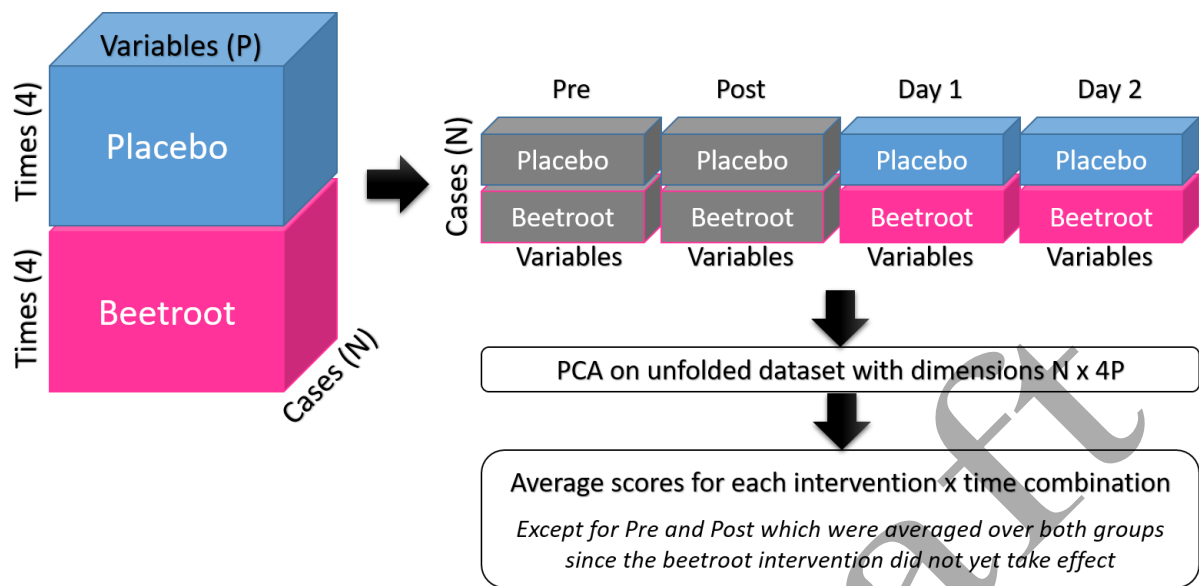


Fig. S1: A summary of the unfolded PCA of the beetroot and placebo interventions over time. Abbreviations: *N*: number of cases; *P*: number of variables.

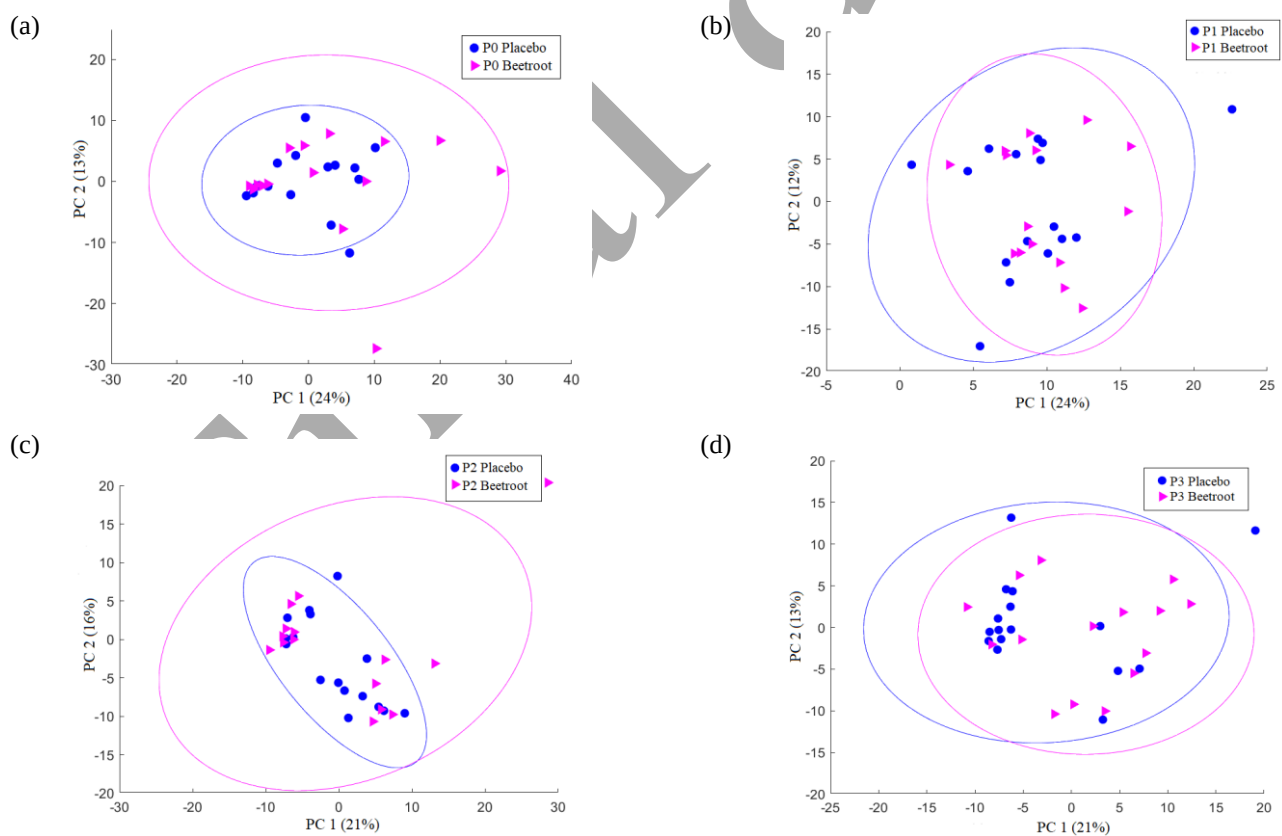


Fig. S2: Principal component analysis plots representing the inter-cohort differentiation of (a) pre-marathon [P0], (b) post-marathon [P1], (c) 24 h post-marathon [P2], and (d) 48 h post-marathon [P3] metabolic profiles. Abbreviations: PC: principal component.